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THE ANTIMICROBIAL ROLE OF HUMAN MAIT CELLS

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Cover illustration: Illustration of the thesis findings. Beavers are depicted as the APCs, who catch more successfully IgG-opsonized bacteria than non-opsonized one. Beavers that feed on IgG-opsonized bacteria are more efficient to activate MAIT cells (magenta cell) due to increased MR1 antigen presentation. Activated MAIT cells secrete cytokines (purple and red arrows) and cytolytic proteins such as granzyme B and granulysin (green and blue arrows respectively) that damage the membrane of carbapenem resistant *E. coli* (purple bacteria). Due to bacterial antibiotic resistance by membrane impermeability, carbapenem antibiotics (white dots casted by the little dragon) stay outside of *E. coli*. However, granzyme B and granulysin synergize with carbapenem to restore the bactericidal activity of the antibiotic. Finally, *S. aureus* (orange cocci) secrete LukED toxin (cadmium red dots) that can kill MAIT cells. By Caroline Boulouis.

The antimicrobial role of human MAIT cells

THESIS FOR DOCTORAL DEGREE (PhD)

By

Caroline Boulouis

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To all the encounters that lead me to this path

Qui cherche trouve

Proverbe familial

ABSTRACT

MAIT cells are unconventional T cells that have characteristics of both innate and adaptive immunity. Their fast mobilization and wide distribution in tissues make them part of the first line of defense against infection. MAIT cells recognize riboflavin-related metabolites produced by bacteria and presented by MR1. However, they can also sense viral infection through non-specific activation by cytokines. MAIT cells respond rapidly with secretion of cytokines and degranulation of cytolytic molecules, and play an important role in immune defense. This thesis work aimed to further explore the function of MAIT cells in different contexts.

First, we explored the interaction between humoral IgG responses and MAIT cells in antibacterial immunity. Here, MAIT cell responses to IgG-opsonized bacteria were compared to responses against non-opsonized bacteria. MAIT cell responses against opsonized *Escherichia coli* were stronger, with an increased magnitude and faster kinetics. Furthermore, MAIT cells were activated at lower bacterial doses when opsonized. We deciphered the mechanism responsible for the MAIT cell boost of function and showed that FcγR triggering by the opsonized bacteria was essential to increase MR1 antigen presentation. The boost of function was validated in a vaccine setting, where we used sera from individuals before and after vaccination against *Streptococcus pneumoniae* to opsonize one vaccine strain. MAIT cell functions were boosted when stimulated with *S. pneumoniae* opsonized with sera drawn after vaccination. In the second project, we investigated the cytolytic mechanisms used by MAIT cells against *E. coli*. Interestingly, MAIT cells not only killed *E. coli*-infected cells but also controlled cell-associated bacterial load through degranulation. More precisely, Granzyme B (GzmB), Granulysin (Gnly), and perforin were involved in the killing. MAIT cell activity was maintained against carbapenem-resistant *E. coli* (CREC). CREC strains use two main mechanisms of resistance: production of carbapenemases that inactivate carbapenem antibiotics, and membrane impermeability by porin-loss or over-expression of efflux pumps that block antibiotic penetration in the bacteria. Interestingly, MAIT cell-derived Gnly and GzmB were able to damage the membrane of free-living CREC. This effect was further enhanced by the addition of carbapenem antibiotic, suggesting a synergy between the cytotoxic proteins and the antibiotic. This also implied that MAIT cells may overcome the impermeability mechanism of resistance of CREC. In the third project, we investigated if some bacteria can adapt to escape from MAIT cell responses through immune evasion mechanisms. *Staphylococcus aureus* produces the pore-forming toxin leukotoxin ED (LukED) that binds to CCR5. We found that MAIT cells were hypersensitive to LukED and this was due to very high expression of CCR5 on MAIT cells. Within the T cell pool, MAIT cells were the most severely depleted population indicating that LukED secretion constitutes an immune evasion mechanism from MAIT cell recognition.

In conclusion, these findings indicate that MAIT cell responses are boosted against IgG-opsonized pathogens. Furthermore, MAIT cell cytolytic activity is maintained against CREC, and GzmB and Gnly synergize with carbapenem antibiotics to kill free-living CREC. Finally,

some bacteria have developed means to evade MAIT cell responses, and this includes LukED secretion by *S. aureus*, which strongly targets MAIT cells. Overall, this thesis work enhances our understanding of MAIT cells as important antimicrobial immune cells.

LIST OF SCIENTIFIC PAPERS

I. **Boulouis C**, Gorin JB, Dias J, Bergman P, Leeansyah E, Sandberg JK. Opsonization-Enhanced Antigen Presentation by MR1 Activates Rapid Polyfunctional MAIT Cell Responses Acting as an Effector Arm of Humoral Antibacterial Immunity. J Immunol. 2020 Jul 1; 205(1):67-77.

II. **Boulouis C***, Sia WR*, Gulam MY, Teo JQM, Png YT, Phan TK, Mak JYW, Fairlie DP, Poon IKH, Koh TH, Bergman P, Lim CM, Wang LF, Kwa ALHº, Sandberg JKº, Leeansyah E. Human MAIT cell cytolytic effector proteins synergize to overcome carbapenem resistance in *Escherichia coli*. PLoS Biol. 2020 Jun 8; 18(6), e3000644.

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SIV. Leeansyah E, **Boulouis C**, Kwa ALH, Sandberg JK. Emerging Role for MAIT Cells in Control of Antimicrobial Resistance. Trends Microbiol. 2020 Dec 19:S0966-842X(20)30314-0.

SV. Leeansyah E, Hey YY*, Sia WR*, Ng JHJ, Gulam MY, **Boulouis C**, Zhu F, Ahn M, Mak JYW, Fairlie DP, Kwa ALH, Sandberg JK, Wang LF. MR1-Restricted T Cells with MAIT-like Characteristics Are Functionally Conserved in the Pteropid Bat Pteropus alecto. iScience. 2020 Nov 28;23(12):101876.

SVI. Parrot T, Healy K, **Boulouis C**, Sobkowiak MJ, Leeansyah E, Aleman S, Bertoletti A, Sällerg-Chen M, Sandberg JK. Expansion of donor-unrestricted MAIT cells with enhanced cytolytic function suitable for TCR-redirection. JCI Insight. 2021 Feb 9:140074

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LIST OF ABBREVIATIONS

Ac-6-FP	Acetyl-6-formyl pterin
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen presenting cells
AMP	Antimicrobial peptides
BAL	Bronchoalveolar lavage
BCG	Bacillus Calmette-Guerin
Bcl6	B-cell lymphoma 6 protein
BCR	B cell receptor
Blimp1	B-lymphocyte-induced maturation protein 1
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CM	Central memory
CFU	Colony-forming unit
CRE	Carbapenem-resistant *Enterobacteriaceae*
CREC	Carbapenem-resistant E*scherichia coli*
CS&T	Cytometer setup & tracking
CTV	Cell Trace Violet
CXCL	Chemokine (C-X-C) ligand
CXCR	CXC chemokine receptor
DARC	Duffy antigen receptor for chemokines
DC	Dendritic cell
DCM	Dead cell marker
DN	Double negative (as CD4-CD8-)
DR3	Death receptor 3
E. coli	*Escherichia coli*
EGTA	Ethylene glycol tetraacetic acid
Eomes	Eomesodermin
ER	Endoplasmic reticulum

ESBL	Extended spectrum β-lactamase
FACS	Fluorescence-activated cell sorting
FcγR	Fc γ receptor
FSC	Forward-scatter
F. tularensis	*Francisella tularensis*
GM-CSF	Granulocytes-macrophage colony stimulating factor
Gnly	Granulysin
GzmA	Granzyme A
GzmB	Granzyme B
GzmK	Granzyme K
HLA-DR	Human leukocyte antigen – DR isotype
IC_{50}	50% of minimum inhibitory concentration
ICOS	Inducible T-cell costimulator
IFN	Interferon
IL	Interleukin
ILC	Innate lymphoid cell
ILXR	Interleukin receptor for interleukine X (X=number)
IgG	Immunoglobulin G
IMP	Imipenemase
IVIg	Intravenous Immunoglobulin G
KIR	Killer-cell immunoglobulin-like receptor
K. pneumoniae	*Klebsiella pneumoniae*
KPC	*Klebsiella pneumoniae* carbapenemase
LB	Lysogeny broth
LPS	Lipopolysaccharide
LukED	Leukotoxin ED
MAIT cell	Mucosa-associated Invariant T cell
MDR	Multidrug-resistant
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration

MRSA	Methicillin resistant *Staphylococcus aureus*
MR1	MHC-Ib related
MR1T cell	MR1-restricted T cell
MTB	*Mycobacterium tuberculosis*
MVC	Maraviroc
NDM	New Delhi metallo-β-lactamase
NIH	National Institutes of Health
NK cell	Natural killer cell
NO	Nitric oxide
NP	Nasopharyngeal
OXA-48	Oxacillinase-48
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood monocular cells
PBP	Penicillin binding proteins
PD-1	Programmed cell death protein 1
PLZF	Promyelocytic leukemia zinc factor
PMT	Photomultiplier
PRR	Pattern recognition receptor
Prf	Perforin
RORγT	Retinoid-related orphan receptor γT
ROS	Reactive oxygen species
SARS-Cov2	Severe acute respiratory syndrome coronavirus-2
SEB	Staphylococcal enterotoxin B
S. aureus	*Staphylococcus aureus*
SIV	Simian immunodeficiency virus
S/n	Supernatant
S. pneumoniae	*Streptococcus pneumoniae*
SSC	Side-scatter
STAT3	Signal transducer and activator of transcription 3
S. Typhi	*Salmonella enterica* subsp *enterica* serovar Typhi
S. Paratyphi A	*Salmonella enterica* subsp *enterica* serovar Paratyphi A

T-bet	T-box transcription factor 21
TCR	Effector memory T cells
TEM	T cell receptor
TEMRA	Terminally differentiated effector memory CD45RA+ T cells
Tfh	T follicular helper
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domain
TL1A	TNF-like protein 1A
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T
UMAP	Uniform manifold approximation and projection
VIM	Verona integron-encoded metallo-β-lactamase
XDR	Extensively drug-resistant
5-A-RU	5-amino-6-D-ribitylaminouracil
5-OE-RU	5-(2-oxoethylideneamino)-6-D-ribitylaminouracil
5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6-FP	6-formyl pterin

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM IN THE LIGHT OF MAMMALIAN EVOLUTION

Mammals, including humans, have developed tools and mechanisms to protect us against many threats. The immune system is a formidable network able to defend us against microorganisms (bacteria, viruses, fungi and parasites), the toxins they produce and the damage they can make. The immune system can also fight internal threats such as cancerous cells. This network has evolved over millions of years to achieve the level of sophistication it has reached in humans and other present-day mammals. Diverse features have been integrated through evolution and those are briefly described in the next paragraphs.

Classically, the immune system is divided into two branches: the innate and adaptive branches. The first reacts rapidly and non-specifically, while the second recognizes specific epitopes and allows building of long-lasting immune memory. Most of the living organisms on Earth are relying on the innate immune system for defense against infectious threat (1). This is not limited to the animal kingdom but also extends to prokaryotes (2), plants (3, 4), and is also present in fungi (5). The innate immunity of invertebrates (insects, mollusks, etc) relies on cellular events such as phagocytosis of foreign material, and the secretion of antimicrobial peptides (AMPs) that carry antibacterial or antifungal properties, and differs between species. Additional mechanisms include the production of reactive-oxygen species (ROS) and nitric oxide (NO). The discrimination between the host and the invader is achieved through the sensing of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, unmethylated CpG DNA by the pattern recognition receptors (PRR). The PRRs are germline encoded and include Toll receptors, which are the equivalent of Toll-like receptors (TLR) in mammals (1, 6-9). PRR triggering leads to activation of host defense mechanism. This system allows recognition of broad groups of pathogens but is unable to discriminate between different strains. Vertebrates have similar innate detection systems (6).

The first hint of adaptive features appeared in jawless vertebrates with the development of a system to recognize diverse antigens using variable lymphocyte receptors (1). 400 million years ago, a breakthrough event happened in vertebrates: the appearance of the adaptive branch of immunity with the capacity to recognize specific antigens and the formation of long-term memory cells. B (bursa of Fabricius) and T (thymus) lymphocytes are the two main cellular lineages of adaptive immunity (10). The capacity of the B cell receptor (BCR) and T cell receptor (TCR) to undergo V(D)J recombination provide a diverse repertoire with capacity for specific antigen recognition. Following an infection, some T and B cell clones will be retained as memory cells that can be reactivated faster in the event of re-infection. Another major event in vertebrate immunity is the capacity to discriminate self versus non-self introduced by the major histocompatibility complex (MHC), which present antigens to conventional T cells. This advancement comes with the price of potential self-reactivity (10).

1.2 THE HUMAN IMMUNE SYSTEM

Anatomic and chemical barriers such as skin and mucosal surfaces are the fist line of defense against infection. Upon pathogen breach of these barriers, the cellular component of the innate immunity takes the lead of the response. Local macrophages and dendritic cells (DCs) are able to take up bacteria through phagocytosis and can kill ingested pathogens. Upon PRR triggering, those cells secrete diverse cytokines, chemokines, and AMPs that contribute to local inflammation, immune cells recruitments, and pathogen killing (Luster, 2002). Within minutes, neutrophils, which are crucial effector cells, are recruited to the site of infection followed by monocytes (11, 12). The innate branch also includes innate lymphoid cells (ILCs) that amplify the inflammatory signals, and natural killer (NK) cells that have cytotoxic properties and can lyse infected cells (13, 14). Lastly, the complement system, composed of soluble proteins present in blood and fluids, helps pathogen clearance upon activation (15). The immune response is tailored according to the kind of pathogen our body encounters and fine-tuned to the danger signals activated by the threat.

While the innate immune cells may be able to clear pathogens, in some cases they need help from the adaptive arm of the immune system. Establishment of the adaptive immune response takes time, usually 7-10 days, and the innate immunity has to hold until adaptive lymphocytes come to the rescue (7). Communication and interaction between the innate and adaptive branches is essential for the downstream response. DCs are professional antigen-presenting cells (APC) that can migrate to secondary lymphoid tissues (lymph node, spleen, Peyer's patches) to present foreign antigen to naïve T cells. Specific clones will go through clonal expansion and differentiate into effector T cells before infiltrating the site of infection (16). Effector T cells are short-lived but some will remain as long-lived memory T cells. B cells get activated with the help of follicular DCs and T follicular helper (Tfh) cells and differentiate into plasma cells for antibody production or memory B cells. Affinity maturation is an important step happening in the germinal center of the lymph node to improve affinity of antibodies for their cognate antigen (17).

On the border between the innate and adaptive branches of cell-mediated immunity we find the family of unconventional or innate-like T cells, which display both innate and adaptive characteristics. It comprises γδ T cells, CD1-restricted T cells such as invariant natural killer T (iNKT) cells, and MR1-restricted T cells primarily including the Mucosa-Associated Invariant T (MAIT) cells (18-20). Contrary to classical T cells, the innate-like T cells recognize non-peptide antigens and are donor-unrestricted. Their abundance means they can rapidly respond in large numbers and sustain protection of the host while the adaptive immune response is still maturing (18).

1.3 MUCOSA-ASSOCIATED INVARIANT T (MAIT) CELLS

MAIT cells are perhaps the most abundant and significant subset of unconventional T cells, and are the primary focus of this thesis.

1.3.1 Towards ligand discovery

MAIT cells express a semi-invariant αβTCR. The TCR α-chain invariably contains the Vα7.2 segment and is highly conserved between individuals. Vα7.2 is encoded by TRAV1-2 and combined with a limited set of J (TRAJ 12/20/33) segments. The TCRα chain is paired with a limited Vβ repertoire. Thus, the MAIT cell TCR diversity is very limited compared to the huge repertoire of conventional T cells (21-23). The MAIT cell TCR recognize small microbial metabolites presented by the MHC-Ib-related protein (MR1). The most potent activating ligands known to date are the vitamin B2 (riboflavin)-related metabolite antigens (24). Before continuing with a more in-depth description of MAIT cell ligands below, let us briefly touch upon the evolutionary aspects of the recognition of such metabolites. Riboflavin is necessary for the basic metabolism of animals and microorganisms. The riboflavin biosynthesis pathway is conserved in many microbes, whereas it is not present in mammals (or any animals). Bacteria that do not synthetize riboflavin must be able to take it up from the local environment (as riboflavin is critical for bacterial survival and growth) and also have the advantage of largely avoiding MAIT cell TCR recognition (25-27).

The folic acid (vitamin B9) metabolite 6-formyl pterin (6-FP) was the first MR1 ligand discovered (28), later followed by its acetylated and more stable version Ac-6-FP (24). Vitamin B9 metabolites are able to provoke MR1 egress to the cell surface, but do not activate MAIT cells (24). Vitamin B2 (riboflavin)-related derivatives (28) and metabolites (29) as MR1 ligands were discovered around the same time. The reaction of the metabolite 5-amino-6-D-ribitylaminouracil (5-A-RU) with host-derived methylglyoxal or glyoxal create the unstable antigen 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and 5-(2-oxoethylideneamino)-6-D-ribitylaminouracil (5-OE-RU). These unstable pyrimidines bind to MR1 trough covalent Schiff base formation (29). If left unbound, the pyrimidine intermediates are converted into ribityl lumazine that weakly activates MAIT cells without Schiff base formation (28, 29). A crucial component of the activating ligand is the presence of the ribityl chain that is essential to bind the MAIT TCR and activate MAIT cells (29, 30). In addition to these natural MR1-presented ligands, synthetic drugs can also bind to MR1. Diclofenac metabolites potently activate MAIT cell with neither Schiff base bond formation nor upregulation of MR1 cell surface expression. On the other hand, salicylates are potent competitive inhibitors, nearly as potent as Ac-6-FP (31). A recent study (32) found a new mechanism of MAIT cell antagonism. Through an *in silico* screen, the compound DB28 (3-([2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4yl]formamido)propanoic acid) was identified as a potent non-microbial antagonist able to reduce MR1 cell surface expression by blocking MR1 egress from the endoplasmic reticulum (ER). DB28 is not able to form Schiff base with MR1 but stabilize it through hydrophobic interactions.

1.3.2 The MR1 antigen presentation pathway

Hashimoto et al. discovered MR1 in 1995 (33). MR1 is thought to have appeared about 170 million years ago and is a highly conserved protein in placental mammals (34-36). Some mammals have lost MR1 during the course of evolution: armadillo, carnivores and

lagomorphs (rabbits) lack MR1 (34). No MAIT cells are found in those species but compensatory mechanisms may exist (34). Human MR1 shares the highest homology with those of non-human primate and is more closely related to several bats MR1 (>80% homology) (37), than to mouse or rat homologs. MR1 is widely expressed in human cells, MR1 mRNA expression was detected in all tissues examined, including PBMC, thymus, lung, liver, heart, brain, small intestine, colon, placenta, ovary, testis, prostate, kidney, pancreas, spleen, and skeletal muscle (33). Unloaded MR1 is retained in the ER with the help of tapasin and tapasin-related protein chaperones, in a conformation open to ligand binding (38, 39). Once MR1 binds a ligand, it traffics to the cell membrane (Figure 1).

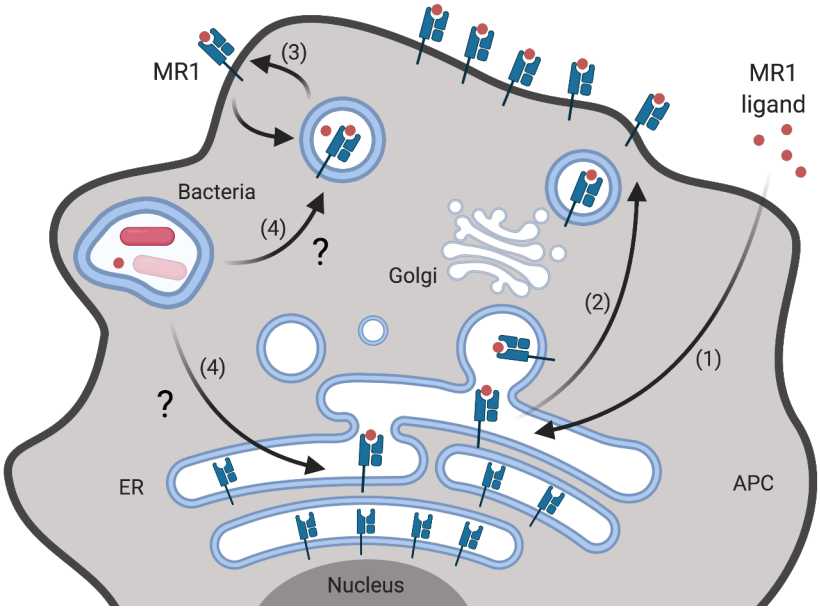

Figure 1. The MR1 antigen presentation pathway. Exogenous riboflavin-related metabolites reach the ER and bind to MR1 molecules through Schiff base formation (1). Loaded MR1 translocates to the cell surface (2) and can be recycled to endosomal compartments where it can be charged with new ligands (3). Ligands derived from intracellular bacterial infection may be loaded in the endosome or in the ER (4). APC: antigen presenting cell; ER: endoplasmic reticulum. Created with BioRender.com.

The translocation signal is given by the Schiff base formation between the Lys43 of MR1 and the ligand. Some ligands bind to MR1 without Schiff base formation, such as diclofenac metabolites, 6-FP and ribityl lumazine. They are less efficient in provoking MAIT cell egress and fail to activate MAIT cell to the same extent as 5-OP-RU (29, 31). It is currently unknown how the ligands reached the ER but both extracellular and intracellular sources of ligands can be loaded on MR1 in this compartment (38, 40). Exogenous ligands reach the ER and bind to MR1 within 10 minutes of exposure, and the resulting complex is then expressed at the cell surface within two hours (39). Ligands derived from intracellular bacterial infection may be preferentially be loaded in endosomal compartments, but this is not fully elucidated yet (40, 41). The MR1-ligand complex remains at the cell surface for several hours. The cell surface expression is dependent on the continual delivery of new MR1-loaded ligand, which is different from MHC class I molecules whose the residence time at the plasma membrane depends primarily on the ligand affinity. Thus, when the pathogen is

cleared and the ligand levels drop, the MR1 surface expression decreases (42). Once MR1 is internalized, it can either be degraded or acquire new ligands in the recycling pathway (38, 40, 41, 43). This step may occur in the endo/lysosomal compartment since the removal of the Schiff base bond requires low pH (44). This tight mechanism of regulating MR1-cell surface availability constitutes a real-time scanning mechanism by the APC for antigens that can be detected by MAIT cells (42).

Two MAIT cell deficient patients have thus far been described (45, 46). Howson et al (46) found a homozygous point mutation Arg31His in MR1 that impaired riboflavin-related metabolite ligands binding to MR1. Folic acid (pterin)-based ligands such as Ac-6-FP were still able to bind this mutated MR1 and provoke its egress at the cell surface. The patient was devoid of circulating MAIT cells, but seemed to compensate this deficiency with an expanded γδ T cell population (46).

1.3.3 Identification of MAIT cells by flow cytometry

MAIT cell identification was initially done by flow cytometry with the expression of CD3, Vα7.2 and CD161. This was commonly accompanied with CD26, CD8 and IL-18R, which are also highly expressed by MAIT cells (47, 48). Once the MAIT cell antigens were identified, this allowed the development of new technology able to accurately identify MAIT cells: MR1 tetramer loaded with 5-OP-RU (49). This reagent became available to the field in 2016 through the NIH Tetramer Core Facility.

1.3.4 MAIT cell localization and residency

MAIT cells and bacteria have co-evolved through mammalian evolution, such that MAIT cell distributions across tissues seems to reflect the presence of the microbiota and exposure to microbial pathogens (34, 50). MAIT cells are enriched at different barrier sites, including mucosal tissues: in the oral mucosa (51), all along the gastrointestinal tract (47, 49, 52-54), in the liver where they can be up to 50% of T cells (47, 54), in the lungs (55), in the skin (56) and in the female genital tract (57). MAIT cells mostly lack expression of CCR7 (47), an important receptor for entry into secondary lymphoid organs (58) and therefore are scarce in those sites, including lymph nodes (47), but they are present in the thoracic duct lymph (59). In blood, MAIT cells constitute 1-10% of the circulating T cell population of healthy donors (47). It is interesting to note that the MAIT cell frequency in circulation varies quite substantially between healthy humans. The basis for this is not clear, but may reflect exposure to distinct microbiota as well as infection history. It could also be linked to genetic background and inherited or inborn error of immunity.

Peripheral blood MAIT cells have tissue-homing properties and this reflects their distribution in the body (Figure 2). CCR9 and CCR6 guide their homing to the gut, CXCR6 to the liver (47), CXCR3 to the lung (59) and CCR5 (47) and CCR2 to inflamed tissues (60). MAIT cells also express CCR4, which may confer skin-homing capacity (61), and CXCR4 (47). Recently, the expression of CXCR5, which supports homing to germinal centers, was

detected at low levels in blood MAIT cells (62), suggesting their possible migration to such lymphoid tissue sites.

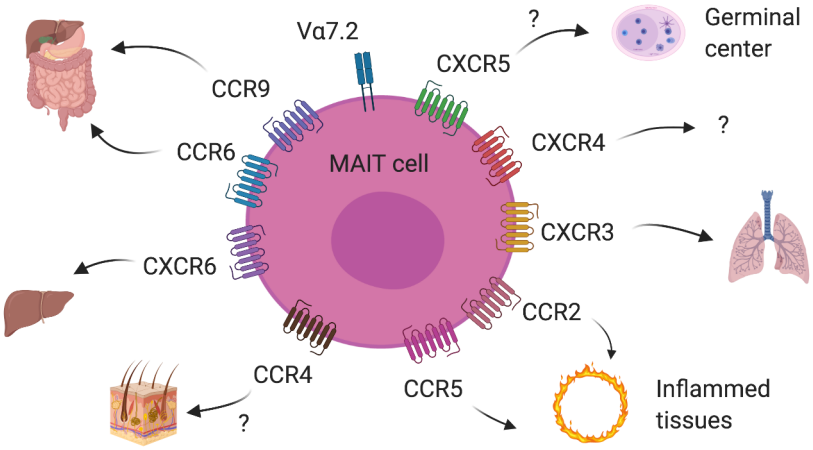

Figure 2. Chemotaxis properties of MAIT cells. Chemokine expression on blood MAIT cells and their tissue homing capacities. Created with BioRender.com.

How and when MAIT cells are seeded in tissues and if MAIT cells form a true tissue-resident population is still not entirely clear. Parabiosis experiments in mice show that tissue MAIT cells do not recirculate (63, 64). Deciphering if the same applies to humans remains to be explored. One study found that human MAIT cells express a stronger tissue residency program in the liver compared to blood and that the tissue localization affects gene expression (64), while another study did not find such MAIT cell tissue residency signature in the liver (65). One hypothesis is that tissue MAIT cells may be a mixture of resident and circulating cells (51, 66). In the oral mucosa, the Jα chain usage in MAIT cells was more diverse than their matched blood counterpart (51). Furthermore, MAIT cells are found in the thoracic duct, which drains the lymph coming from nearly all organs in the body. The TCR clonotypes of MAIT cells in the thoracic lymph was found to be similar to those seen in blood. This suggests that they may be able to leave the tissue and re-circulate, or that MAIT cells are able to migrate to lymph nodes in a CCR7-independent manner (59).

1.3.5 MAIT cell development and homeostasis

MAIT cell development and maturation occurs in two main steps: selection in the thymus by MR1-expressing thymocytes, followed by expansion in the periphery. It is likely that the microbiota colonization is crucial for MAIT cell development, since germ-free mice are devoid of MAIT cells (63, 67-71). The exposure to microbiota must occur early in life or MAIT cell will not develop (71). Although it is still unclear if MAIT cells are selected by empty MR1, MR1/5-OP-RU complexes or by self-antigens (72), 5-OP-RU can travel from the periphery to the thymus and bind to MR1 (63, 73). Koay et al (69), described a three-stage development process of human MAIT cells, based on the expression of CD161 and CD27. MAIT cells at development stage 1 are located in the thymus, display a naïve phenotype and lack PLZF expression. Under the control of PLZF, stage 2 MAIT cells migrate outside the thymus to continue their maturation to stage 3 in the periphery. T-bet is

probably also important for MAIT cell development since MAIT cells were found to be very low in numbers in a recently described T-bet deficient patient (74). Some functions such as tissue homing may be imprinted already in the thymus. For instance, MAIT cells have a gradual expression of chemokine receptors CCR5 and CCR6 during thymic maturation (75). Functionally, some stage 3 thymic MAIT cells can produce cytokines but at a lower level than the stage 3 cells in blood (69), highlighting the importance of peripheral maturation. In humans, the seeding of MAIT cells in tissues may occur before birth, since they have been found in small intestine, liver, and lung of second trimester fetal tissues (76). At birth, the percentage of MAIT cells in blood is lower than in healthy adults (68, 70). After birth, the MAIT cell population increases gradually with age in the peripheral blood (47, 69), having a low expansion rate during infancy to finally reach a plateau around the age of six (70). Within two months after birth, half of the MAIT cells have already acquired a memory phenotype (70). The MAIT cell percentage remains relatively stable in adults, before starting to gradually decline during ageing. The MAIT cell reduction in the elderly is balanced by the expansion of some clonotypes. Furthermore, full functionality of MAIT cells is maintained in aged individuals (77).

MAIT cells express a high level of IL-7Rα (78), the receptor for IL-7, a well-known cytokine for maintenance of lymphocyte populations (79). The importance of IL-7 for MAIT cell development and maintenance is still unknown; although recent findings indicate that recombinant IL-7 treatment in humans can expand the peripheral blood MAIT cell pool (80, 81).

1.3.6 MAIT cell activation phenotype, effector functions and roles in infection

MAIT cells express diverse transcription factors that shape their functionality. T-bet, Eomes and Blimp1 frame their Th1 phenotype and the secretion of IFNγ/TNF along with their cytotoxic capabilities through the release of perforin, granzyme B (GzmB) and granulysin (Gnly) (82, 83). The expression of RORγT and STAT3 underpins their Th17 phenotype and the production of IL-17A and IL-22 (84). PLZF is crucial for MAIT cell development (69), but is also associated to the high expression of IL-12R and IL-18R on MAIT cells (66). MAIT cells also express the transcription factor Helios but its role remains unclear (85).

1.3.6.1 MAIT cell activation

MAIT cell activation occurs by two main ways: TCR-dependent and TCR-independent, the latter being trough cytokine stimulation. These two pathways can also synergize to activate MAIT cells.

TCR triggering is essential for MAIT cell activation but is not sufficient to fully engage all MAIT cell functions (82, 86, 87). To be fully activated, MAIT cells need co-stimulation signals, via CD28, TLRs or cytokines (88-90). Indeed, in mice, the administration of 5-OP-RU alone in the absence of TLR agonists does not induce MAIT cell proliferation, activation or accumulation in the lung (90, 91). In regard of the broad expression of MR1, this

mechanism of regulation is essential to avoid over activation towards components of the microbiota that also produce riboflavin metabolites (66). Thus, MAIT cells are "poised to maintain homeostasis in the presence of commensals" (90).

Cytokines can activate MAIT cells in an MR1-independent manner. MAIT cells express a high level of IL-12R (65, 86), IL-18R (27), IL-15R, IFNαR (65), and DR3 (92), and are activated by their ligands, respectively IL-12, IL-18, IL-15, IFNα/β and TNF-like protein 1A (TL1A). IL-12 and IL-18 combination was first described to activate MAIT cells without TCR triggering (93). Later, IL-15 (94) and type I interferons (IFNα/β) (95, 96) in synergy with IL-18, and TL1A (87) in combination with IL-18, IL-12 and IL-15 were also found to activate MAIT cells. Cytokine-mediated activation narrows the MAIT cell functional profile to the secretion of IFNγ and GzmB (96).

Synergy between TCR triggering and cytokine activation brings the full range of MAIT cell functions (54, 61, 86, 87, 89, 93, 97), which is the case in bacterial (83, 98) and fungal (85, 99, 100) infections. TCR-dependent activation is known to occur within 6 hours of bacterial stimulation, while the synergy of TCR and cytokine activation happens later (93). Once fully activated, MAIT cells express several activation markers such as CD69, CD25, CD38, HLA-DR (27, 101, 102), and produce a wide array of effector molecules. Upon *in vitro* bacterial stimulation, blood MAIT cells secrete IFNγ, TNF, IL-17A and release the cytolytic proteins GzmB, perforin, Gnly (57, 83, 98, 103). MAIT cells can also secrete chemokines, including CCL3, CCL4, CCL20, CXCL9, CXCL10, CXCL11 and IL-8 (CXCL8) (78, 86, 104).

MAIT cells can be activated by numerous types of APCs: monocytes, macrophages, DCs, and B cells but the responses will differ depending on the type of APC (98, 105-107). Stimulation with non-professional APCs, such as epithelial cells, will drive mainly TCR-dependent responses (108).

Cytokine production in tissue MAIT cells differs from their blood counterparts (50, 51, 53, 57, 65, 78, 97, 109). Tissue MAIT cells have a more pronounced Th17 phenotype with high production of IL-17 in the bronchoalveolar lavage (BAL) of pneumonia patients (110), in the female genital tract (57), and in the oral mucosa (51) of healthy individuals. In the liver, MAIT cells are more responsive to IL-12 and IL-18 than in the blood (65).

1.3.6.2 Cytotoxic properties of MAIT cells

MAIT cells are well known for their ability to kill bacteria-infected cells in a TCR-dependent manner (82, 83, 103) (Figure 3). Cytotoxic lymphocytes share a conserved mechanism of killing target cells: the release of granules containing numerous cytotoxic proteins (111). Upon appropriate stimulus such as antigen detection, the granule content (perforin, granzymes and Gnly) will be released into the synapse formed with the target cell. Perforin, a pore-forming toxin is first needed to perforate holes and hence permeabilize the target cell membrane. It allows the granzymes and Gnly to enter the cytoplasm and promote cell death by apoptosis (111). Granzymes are serine proteases that cleave specific substrates, unique for each granzyme subtype (112). MAIT cells express GzmB, GzmA and GzmK (83). GzmB is a

potent pro-apoptotic granzyme that cleaves many essential mammalian proteins (111). It also targets bacterial enzymes and proteins necessary for oxidative stress defense, metabolism and protein synthesis, thus acting as a powerful antibiotic (113, 114). GzmB can also digest bacterial toxins (Leon, 2020). GzmA and GzmK have pro-inflammatory properties, although GzmA may also induce apoptosis through caspase-3-independent pathway (111, 112). Gnly is a saposin-like protein that creates holes in cholesterol poor membrane, thus it preferentially targets bacteria rather than human cells. It allows GzmB penetration into the bacteria, unleashing its antimicrobial properties (114). Rodents are devoid of Gnly, thus MAIT cell antimicrobial activity cannot be fully assessed in murine models (115).

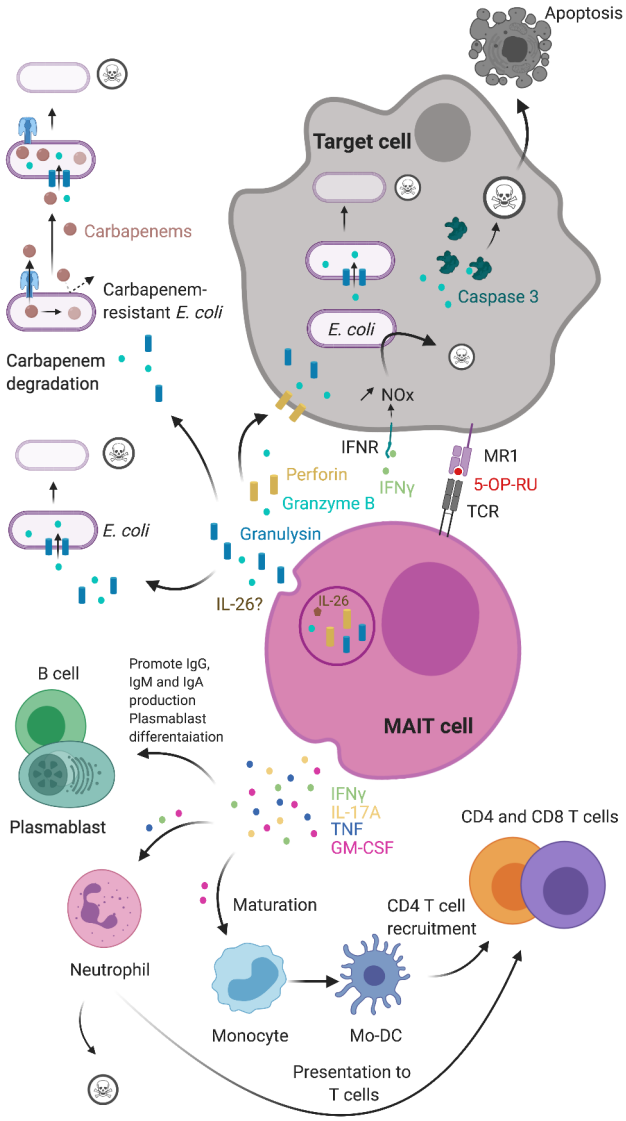

Figure 3. Antimicrobial properties of MAIT cells (from (116)). MAIT cells produce cytokines that help recruit other immune cells, induce immune cell maturation and directly control bacterial load. Cytotoxic molecules released by MAIT cells kill infected cells but also kill intracellular and extracellular bacteria, including carbapenem-resistant ones (see paper II). Created with BioRender.com.

At resting steady state, MAIT cells express GzmA and GzmK but only low levels of perforin and Gnly, and little GzmB (83, 103). Upon activation, MAIT cells upregulate GzmB, perforin and Gnly and degranulate as seen with surface staining of CD107a. Importantly, MAIT cells

can be armed in response to different stimuli. Indeed, MAIT cells pre-activated with IL-7 (82), fixed bacteria (83) or 5-OP-RU in presence of IL-7 and IL-2 (paper II; (117)) display higher cytotoxic capacity than resting MAIT cells.

MAIT cells also secrete IL-26 upon TCR and cytokine activation (87, 118), a potent antimicrobial cytokine, which is able to puncture bacterial membranes and provoke bacterial cell death (119). MAIT cells also secrete CCL20, CCL9 and CXCL10 that display antimicrobial properties (120-122). It is still unknown how these proteins participate in MAIT cell antimicrobial defense.

1.3.6.3 MAIT cells in bacterial and viral infection

MAIT cells play a major role in fighting bacterial infections, as seen *in vivo* or *in vitro* (20, 116). Figure 3 summarizes the antimicrobial functions of MAIT cell. In animal models, MAIT cells protect against infection and control bacteria burden against *Legionella longbeachae*, in a mechanism dependent on IFNγ, GM-CSF and MR1 (123). MAIT cell control of infection was also shown in *Francisella tularensis, Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (MTB) infection (124-127). In mice, MAIT cell-derived IFNγ helps to clear intracellular infection through the induction of nitric oxide in macrophages (Figure 3) (124, 125). MAIT cells can also expand at the site of infection (91, 123, 124). Interestingly, upon vaccination with 5-OP-RU in combination with TLR agonists or IL-23, MAIT cells expand and confer an early protection against *Legionella* infection (91, 123, 128). In contrast, vaccination with 5-OP-RU did not improve the control of acute MTB infection, while its application during chronic infection helps MAIT cell recruitment and bacterial control through IL-17A (126). Thus, MAIT cells may have a potential role in vaccination. MAIT cells are also able to coordinate the response to pathogens. During *F. tularensis* infection of the lung, MAIT cells produce GM-CSF that induces the differentiation of CCR2+ monocytes into monocyte derived-DC that in turn recruit CD4+ T cells (129). Finally, a detrimental role of MAIT cells in infection has also been proposed. In *Helicobacter pylori* infection, MAIT cell accumulated in the gastric mucosa and worsened the outcome of the disease (52). Furthermore, the role of MAIT cells in MTB is ambiguous due to inconsistent findings in the control of MTB infection by MAIT cells in different animal models (125, 126, 130).

In humans, MAIT cells are activated by bacteria producing riboflavin, such as *Escherichia coli* (27, 98), *K. pneumoniae*, *Staphylococcus aureus* (27), *Streptococcus pneumoniae* (105, 131), *Salmonella spp* (101, 102, 132), and *Neisseria gonorrhoeae* (133). MAIT cells often tend to decrease in peripheral blood during infection (27, 55, 101, 102, 134-136), except in some cases where they remain stable (137, 138). Challenge studies with *Salmonella enterica* subsp *enterica* serovar Typhi (*S.* Typhi) (102) and *Salmonella enterica* subsp *enterica* serovar Paratyphi A (*S.* Paratyphi A) (101) showed that blood MAIT cells declined in numbers and were activated upon infection. The secretion of cytokines by MAIT cells is key in some settings. IFNγ and TNF responses by MAIT cells prevent nasal

colonization of *S. pneumoniae* (139). Furthermore, MAIT cell-derived IFNγ is important for MTB immunity (74).

MAIT cell cytokine production also facilitates the recruitment of other immune cells, and further activates neighboring cells that ultimately defeat the infection ((116); Figure 3). First, MAIT cells probably have the ability to help B cells. The supernatant of activated MAIT cells stimulates B cell production of IgG, IgA and IgM and supports the differentiation of memory B cells into plasmablasts (140). A follow-up study found the presence of CXCR5+ MAIT cells in tonsils next to the germinal centers, exhibiting a Tfh phenotype (PD-1+, ICOS+, Bcl6+ and IL-21 secretion) (62). Furthermore, animal studies showed that MAIT cells provide help to B cells through cytokine secretion and stimulate pathogen-specific antibody production (62, 141, 142), as seen *in vitro*. MAIT cells also induce maturation of monocyte-derived and primary DC via the CD40-CD40L axis (106). Finally, MAIT cells interact with neutrophils, although the two studies on this topic are not consistent, probably due to differences in the experiment protocols. Davey et al. (143) found that after antigen presentation by the neutrophils, MAIT cells secrete IFNγ, TNF and GM-CSF that can make neutrophils cross-present antigens to CD8+ and CD4+ T cells. In contrast, Schneider et al. (144) found that neutrophils can suppress MAIT cell activation and that highly activated MAIT cells kill neutrophils.

Human primary immune deficiency brings insights regarding the important roles of MAIT cells in antimicrobial immunity. An individual lacking MAIT cells due to mutations in MR1 was recently identified (46). Despite compensatory increase of γδ T cells population, the individual had a history of unexplained infections revealing immunodeficiency: treatment-refractory *Campylobacter* infection, development of secondary bacterial pneumoniae after viral infection, and human papillomavirus activation after being tattooed. Thus, MAIT cell absence weakens immune protection. In the case of STAT3 deficiency, an important factor of Th17 differentiation, MAIT cells are characterized with impaired IL-17A and IL-17F production while the level of IFNγ and TNF are normal (84). STAT-3 deficient patients are susceptible to pathogens for which Th17 immunity is crucial including *Candida albicans* and *S. aureus* (84) and both these microbes activate MAIT cells (27, 98). Of note, MAIT cells express high level of IL-23R, and IL-23 is important for the maintenance of STAT3 expression in MAIT cells, thus maintaining their Th17 phenotype (84).

Viruses are unable to activate MAIT cells in an MR1-dependent way. However, the release of cytokines during viral infection allows MAIT cell activation, particularly through IL-18 in combination with IL-12, IL-15 and IFNα/β (93, 95, 96, 145). In many viral infections, such as dengue, zika, hepatitis C, hepatitis B and D co-infection, human immuno-deficiency virus (HIV), influenza, measles, and severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), MAIT cells are activated and display an activated phenotype with higher expression of CD25, CD38, HLA-DR, PD-1, CD69, TIGIT (95, 96, 145-153). During such infections, MAIT cell numbers in blood decline, either due to death by apoptosis (146, 152) or recruitment to inflamed tissues, for example, into the lungs as seems to be the case in SARS-

CoV-2 infection (149). In mice, MAIT cells conferred protection against lethal influenza A virus infection, while MR1 deficient mice (MR1-/-) died from the infection (95).

1.3.6.4 Tissue repair function

Recently, at the gene expression level, MAIT cells were found to express a tissue repair transcriptome signature when stimulated through their TCR alone, or TCR combined with cytokines. This is accompanied by the upregulation of genes coding for Furin, CCL3, GM-CSF and certain growth factors (87, 90). The wound-healing activity of MAIT cell was confirmed *in vitro (87)*, and *in vivo* (71). Indeed, 5-OP-RU topical application on mouse skin before wounding was enough to increase MAIT cell repair properties (71). Thus, MAIT cells can promote barrier integrity in homeostasis and healing during tissue injuries (71, 87, 90).

1.3.7 MAIT cell heterogeneity

A T cell clone is defined by a unique TCR sequence. The MAIT cell TCR is composed of the α-chain with very little diversity, paired with a limited β-chain diversity (21-23, 154). MAIT cells appear to have slightly different functional responses to microbes according to the TCRβ chain diversity (85). Moreover, during challenge with *S.* Paratyphi A in human volunteers, there is an evolution of the MAIT cell TCRβ repertoire. Some clonotypes expanded, were more activated during the infection and displayed higher avidity than the others (101). Interestingly, alterations in the MAIT cell clonotype distribution can occur also during acute viral infection, as recently observed in acute HIV-1 infection (155).

MAIT cells express some NK cell-associated receptors such as CD56, CD84, CD94, NKG2D and NKp80. MAIT cells expressing CD56 or CD84 displayed a different transcription factor profile and respond more strongly after IL-12 and IL-18 stimulation (85).

Finally, MAIT cells express CD8 or CD4, and can also be double negative for these markers (DN). CD4+ MAIT cells are a very rare population. The CD8+ MAIT cell cytokine response is stronger than that of DN MAIT cells. CD8+ MAIT cells downregulate CD8 over time during *in vitro* culture. *In vivo*, CD8+ MAIT cells accumulate before DN MAIT cells in fetal development. Thus, we recently proposed that DN MAIT cells are derived from CD8+ MAIT cells (156).

1.3.8 Other MR1-restricted T cells

MAIT cells are the main representative of MR1-restricted T (MR1T) cells but are not the only one. Atypical MAIT cells are phenotypically distinct and do not express TRAV1-2 but still recognize folic acid or riboflavin metabolites or could be MR1-autoreactive. These cells may also lack PLZF, a central MAIT cell transcription factor, and constitute a very rare population (0.01% in blood). Other MR1T cells have been described and more research is needed to fully understand their role (19, 20, 23, 154).

More recently, MR1 was described to present metabolites from cancerous cells to a T cell clone but the ligand is yet to be identified (157). This may open the path for the discovery of possible endogenous MR1 ligand.

In the next section, we will turn our attention to the bacterial side and particularly to antibiotic resistant bacteria.

1.4 ANTIMICROBIAL RESISTANCE

Antibiotic resistance exists in the wild but the overuse and misuse of antibiotics in the clinic and in the farming industry have led to an exponential growth of antibiotic resistant bacteria all over the world (158, 159). It is a serious threat that claims thousands of human lives each year and could take millions in the near future, particularly in developing countries (158). The economic load is also a concern that will keep growing in the following years (158). Furthermore, if antibiotic drugs do not work anymore, many medical interventions (chemotherapy, surgery, Caesarean-section) will be at risk, undermining our current healthcare system (158). Antibiotic resistance is affecting all classes of antibiotics and the development of new classes is outpaced by the emergence of drug-resistant bacteria. In this thesis-work, we used carbapenem-resistant *E. coli* (CREC) in several experiments. Carbapenems belongs to the β-lactam family of antibiotics.

1.4.1 β-lactam antibiotic and their enzymatic degradation

β-lactam antibiotics mediate bactericidal activity by binding irreversibly to penicillin binding proteins (PBP) involved in peptidoglycan synthesis and thus inhibit cell wall synthesis. Structurally, the β-lactam ring of the antibiotic molecule is essential for the reaction with the PBP. β-lactams are composed of four main classes: penicillins, cephalosporins, monobactam, and carbapenems that have different spectra of activity. The carbapenem-family includes among others the drugs imipenem, meropenem, and ertapenem (160).

Bacteria produce β-lactamases that hydrolyze the β-lactam ring of the antibiotic, thus rendering it inactive. The enzymes are classified according to their mode of hydrolysis into four groups proposed by R.P. Ambler (161): Ambler class A, B, C and D and are described more in detail in the next section. Penicillinases and cephaloporinases degrade penicillins and cephalosporins, respectively. Extended spectrum β-lactamases (ESBL) degrade all β-lactams, except carbapenems. Carbapenemases inactivate carbapenems and can also degrade penicillins and cephalosporins (162).

Notably, drugs with the capacity to inhibit β-lactamases have been developed. Inhibitors of β-lactamases act as a decoy for β-lactamases that will preferentially bind to the inhibitor instead of the antibiotic by virtue of a higher affinity. None of the inhibitors available can target all the existing β-lactamases. The inhibitors of β-lactamases that are available on the market include clavulanic acid, tazobactam and sulbactam (163, 164) and are combined with β-lactam antibiotics during treatment.

1.4.2 Carbapenem resistant *Enterobacteriaceae*

Enterobacteriaceae are Gram-negative bacteria that belong to the normal microbiota but can cause infections such as cystitis, sepsis and pneumonia. *E. coli* is a main disease-causing agent both in the hospital and in community settings (163), and infections with carbapenem-resistant bacteria are associated with higher risk of mortality (165). Resistance to antibiotics by enzymatic degradation has built up over the years. The treatment of choice of ESBL-producing Enterobacteriacae is carbapenems (166), and this promoted the development of carbapenem resistance. Colistin is a last-resort antibiotic against carbapenem resistant *Enterobacteriaceae* (CRE) but plasmids encoding resistance to colistin has been recently described (167), leaving us with limited treatment options against multidrug-resistant (MDR) or extensively drug-resistant (XDR) strains. CRE are now ranked as a critical priority for research and development in the WHO guidelines (168).

Carbapenem resistance is often paired with non-enzymatic mechanisms of resistance (165). For example, Gram-negative bacteria have an outer membrane that protects them against antibiotics. However, hydrophilic antibiotics can still get through the porins present on the outer membrane. The loss of porins or the overexpression of efflux pumps, able to transport antibiotic outside the bacteria, confers bacterial resistance to antibiotics by impermeability (169). Lastly, mutations or modifications in the binding site of the antibiotic constitute yet another mechanism of resistance (169).

Some ESBL enzymes have weak carbapenemase activity and if combined with bacteria impermeability mechanisms, they can cause phenotypic carbapenem resistance (163). However, this last mechanism constitutes a lower public health threat than carbapenemase-producing bacteria. Carbapenemases are encoded by plasmids that are highly transmissible by horizontal gene transfer between *Enterobacteriacae* and can be responsible of outbreaks (165).

Carbapenemase enzymes fall into the Ambler class A, B and D (165). The enzymes from class A and D contain serine residues in their active sites (160). Amber class A β-lactamases, represented mainly by KPC enzymes, are broad and can be inactivated *in vitro* by the β-lactamase inhibitor clavulanic acid. Amber class D enzymes differ from A because they also cleave oxacillin antibiotic (and thus were named oxacillinase) and comprise OXA-48 family enzymes. Amber class D enzymes do not hydrolyze cephalosporins from the second and third generations (163). Ambler class B enzymes or metallo-β-lactamases use zinc for hydrolysis of the β-lactam ring. They confer a high level of drug resistance to the bacteria when combined with membrane impermeability since they hydrolyze all β-lactams except monobactam and aztreonam. They are inactivated by metal chelators and not by traditional β-lactamase inhibitors, such as clavulanic acid. The main class B enzymes are NDM, IMP and VIM. The different Ambler classes are being predominant in different parts of the world (160, 163).

Carbapenem resistance is a global and concerning threat. *E. coli* is part of the *Enterobacteriaceae* family and express the riboflavin pathway. It is thus plausible that the strains that have developed carbapenem resistance can still be recognized by MAIT cells.

2 AIMS

This thesis work intends to decipher mechanisms of the antimicrobial role of MAIT cells in different settings.

Overall, the aims can be defined as followed.

Paper I: Investigate the MAIT cell functional response against IgG-opsonized bacteria and in vaccination settings.

Paper II: Explore the cytotoxic mechanisms of MAIT cells against *E. coli*, including carbapenem resistant *E. coli*.

Paper III: Examine the effects of the Staphylococcal toxin LukED on MAIT cells.

3 METHODS

For an in-depth description of the methods used, the reader is invited to consult the methods section of each paper. A brief summary of the main methods performed is described in this chapter.

3.1 ETHICAL CONSIDERATIONS

Ethical permits were granted before any studies started.

For blood and tonsils used in paper I and III, informed written consent was obtained from all donors in accordance with study protocols conforming to the Declaration of Helsinki and approved by the Regional Ethics Review Board in Stockholm.

For the serum samples used in paper I, informed written consent was obtained from all donors and the study was performed in accordance with the Declaration of Helsinki and approved by the Regional Ethics Review Board in Stockholm and by the Swedish Medical Product Agency. The study was registered as NCT01847781.

For blood and nasal tissues used in paper II, informed written consent was obtained from all donors in accordance with study protocols conforming to the Declaration of Helsinki and approved by the National University of Singapore Institutional Review Board and by the Singapore General Hospital Institutional Review Board.

3.2 SAMPLES COLLECTION

Blood samples were obtained from healthy donors following the local rules. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on Lymphoprep and either used fresh for MAIT cell purification (paper I, II, III) or frozen for later use (paper II).

Tonsils were collected from healthy donors undergoing tonsillectomy due to sleeping disorder and tonsillar mononuclear cells were obtained as PBMC after a step of mechanical dissociation and filtering (paper I).

Nasopharyngeal samples were obtained from healthy donors undergoing nasal polyp removal (paper II).

3.3 CLINICAL BACTERIAL STRAINS

3.3.1 Clinical *E. coli* strains

E. coli clinical strains used in the paper II are listed in table 1.

MDR bacteria are resistant to at least one drug in at least three antimicrobial categories. XDR bacteria are non-susceptible to at least one drug in all but two antimicrobial agent categories (170).

Strain	Site of culture	Carbapenemase	ESBL	Efflux protein	*mcr-1*	Resistance
EC120	Blood	Non-CRE	-	ND	ND	N/A
EC234	Blood	Ambler class B	+	-	-	MDR
EC241	Sputum	Ambler class A	+	+	-	MDR
EC362	Sore tissue	Ambler class A	+	ND	+	XDR
EC385	Blood	Ambler class D	+	-	-	MDR

Table 1. *E. coli* strains list used in paper II. ESBL: extended spectrum β-lactamase, *mcr-1*: mobilised colistin resistance N/A: not application, ND: no data.

3.3.2 Clinical *S. aureus* strains

The clinical *S. aureus* strains 134, 289, 37, 159 and 160 were used in paper III and were previously characterized (171).

3.4 FUNCTIONAL ASSAYS

3.4.1 MAIT cell activation assay

To activate MAIT cells, MR1-expressing APC were required. The THP-1 cell line was used for that purpose in paper I and III, while in paper II we used 293T-hMR1 cell (293T stably transfected with human MR1 - gift from Ted Hansen), HeLa cells and in some experiments a complete PBMC population. APCs were fed with different mildly fixed strains of bacteria, according to the project's need. The microbial dose varied with the project, and incubation time usually lasted for 3 hours before adding Vα7.2+ cells at a 2:1 ratio, except for PBMC that already contained MAIT cells. Vα7.2+ cells were used as source of MAIT cells and were isolated from PBMC by MACS purification as per the manufacturer's instructions. Cells were co-cultured for 24 hours, with the last 6 hours in the presence of monensin and brefeldin to assess cytokine production by flow cytometry. This method was previously described by Dias *et al* (98).

To assess MAIT cell degranulation, CD107a antibody was added at the beginning of the co-culture. In selected experiments, anti-CD28 was added as a co-stimulatory signal. In some experiments, we blocked TCR or cytokine activation using anti-MR1, anti-IL-12 or anti-IL-18 antibodies.

3.4.2 MAIT cell expansion assay performed in paper II

MAIT cell expansion was performed following two protocols. The first protocol was based on isolated MAIT cells from fresh PBMC by MACS-purification using either the 5-OP-RU-hMR1 tetramer or the Vα7.2 antibody. MAIT cells were then cultured in the presence of IL-2, IL-7, and CD3/CD2/CD28 polyclonal T cell activator or IL-2 and IL-7 alone for 2 to 15 days, according to the need.

The second expansion protocol utilized cryopreserved PBMC. Thawed PBMC were cultured in IL-7, IL-2 and stimulated with 5-OP-RU antigen at day 0, 5, and 10. At day 11, live cells were separated by density gradient centrifugation on Ficoll. At day 15, MAIT cells were checked for purity, and used in functional assays when purity exceeded 70%. Expanded MAIT cells used in the different assays were cultured according to this second protocol, unless indicated.

3.4.3 MAIT cell cytotoxic assays used in paper II

3.4.3.1 Killing assay

To assess MAIT cell killing of infected cells, HeLa or 293T-hMR1 cells were incubated with the appropriate fixed *E. coli* at the microbial dose of 30 for 3 hours. Expanded MAIT cells were then added at an effector to target ratio 5:1 for 24 hours (as described in (82, 98)). 5-OP-RU-pulsed target cells were used a positive control. CD107a antibody was added at the beginning of the assay to assess MAIT cell degranulation over the course of the co-culture. Apoptosis of the target cell was assessed with caspase 3 staining and live/dead cell marker.

To assess MAIT cell killing of cell-associated bacteria, the adherent HeLa cells were infected with live *E. coli* at different microbial doses for 3 hours in antibiotic free media. Infected cells were then washed with medium containing 200 µg/mL gentamicin and incubated for 1 hour to eliminate extracellular bacteria. The cells were then washed with antibiotic free media before adding the expanded MAIT cells at 5:1 ratio for 3 hours. To allow distinction from the target cells, MAIT cells were stained with Cell Trace Violet (CTV) dye before addition to the culture. For enumeration of bacteria, supernatants were collected and the adherent cells were lysed with 0.1% Triton-X for 10 min at RT. To stop the lysis, lysogeny broth (LB) was added and the lysates were plated in duplicated on LB plate and incubated for 24 hours at 37°C before visual counting. In parallel, a duplicate plate was always run to assess MAIT cell degranulation by flow cytometry as described in (117).

3.4.3.2 Preparation of MAIT cell supernatant

Supernatant used to assess killing of extracellular bacteria was prepared as follows. 293T-hMR1 cells were incubated with 2 nM 5-OP-RU in antibiotic free media for 2 hours before adding the expanded MAIT cells at 10:1 ratio for 24 hours. Supernatant of the co-culture was collected by centrifugation and snap-frozen. The protein content was measured with the LEGENDplex human CD8/NK cell panel.

3.4.3.3 Killing of extracellular bacteria

MAIT cell supernatant was incubated with overnight bacterial culture with or without carbapenem antibiotics in flat-bottom 96-well plate for 24 hours at 37°C. Live bacteria were harvested at different times, diluted in LB broth and plated in triplicate in LB plate and incubated for 24 hours at 37°C before enumeration.

3.5 SPECIAL REAGENTS

The 5-OP-RU antigen was prepared as described in (172).

The MR1 tetramer technology was developed jointly by Dr. James McCluskey, Dr. Jamie Rossjohn, and Dr. David Fairlie; and the material was produced by the NIH Tetramer Core Facility as permitted to be distributed by the University of Melbourne.

3.6 FLOW CYTOMETRY

Flow cytometers are fantastic tools developed in the 1960s and are essential for immunological discovery. Flow cytometers allow physical and fluorescence measurements (known as parameters) of millions of single cells. Because the different measurements are done at the same time, it is called multiparameter flow cytometry. Stained cells are loaded into the flow cytometer fluidic system and the hydrodynamic focusing allows the cells to be aligned in the flow cell. When the cell crosses the center of the focused laser beam, also called the interrogation point, information from the excited fluorochrome is collected as emitted light. In the Fortessa or Symphony analyzers from BD Bioscience, the different lasers are installed sequentially so the cells pass in front of 5 different lasers. The light detectors are installed in polygons and routed with optic fiber. The emitted light is detected by the detectors that transform the photon signal into an electrical signal (electron). The signal is further amplified by the photomultipliers (PMT) and the amplification depends on the voltage applied on the PMT. The electrical pulse carries vital information: its height, area and width that will be extracted by the electronic hardware and allow the display of the information in a plot. The cell itself will scatter light that will be harvested in the forward and side detector. Those physical parameters can then be displayed in a plot with the forward-scatter (FSC) and side-scatter (SSC) parameters (173, 174).

The PMT voltage can be changed by the users. Setting optimal PMT voltage is essential to be at the sweet spot of the detector and to have the best separation between positive signal and background to resolve dim populations. Setting correct PMT voltage gain is a world of its own with several guides available for that purpose (175, 176).

Multiparametric flow cytometry comes with the cost of doing what the field calls compensation. A very comprehensive guide about compensation was written by M. Roederer (177). Most of the emission spectra of fluorochromes are not sharp peaks, but are spread over broad wavelengths, and some of the dyes have overlapping emission spectra. The detectors collecting the light have optical filters that are calibrated for a certain wavelength, but it can happen that the light collected does not only come from the intended fluorochrome but

several, due to spectral overlap. This would lead to wrong results unless we remove unwanted signals trough compensation. By acquiring a single fluorochrome at the time, we see the spillover of each fluorochrome into all the channels. The spillover value into each detector is calculated and used by the compensation algorithm to correct for the spillover (173, 174). Fluorescence spillover leads to a reduction of sensitivity in the affected detector. Because the detectors have difficulty to count number of photons when several fluorochromes are hitting it, this creates spreading errors. This measurement error happens on compensated data. Spreading errors make it difficult to separate populations due to the spread of the negative population into the positive one, and indicates that the panel needs further optimization (178).

The maintenance of flow cytometry instruments is essential for good performance (175, 179, 180). The performance of the instrument should be assessed daily to assure flawless performance and collection of high-quality data. At CIM, we rely on the Cytometer Setup and Tracking (CS&T) software developed by BD (175). By running CS&T beads composed of dim, mid and bright beads, this allows a daily measurement of different parameters including coefficient of variation and PMTV that will be compared to reference values. The performance can either indicate a pass, a warning, or fail. In case of troubleshooting needed, this short guide (181) can be helpful.

4 RESULTS AND DISCUSSION

4.1 MAIT CELL RESPONSES TO OPSONIZED BACTERIA

Opsonization is the process to coat pathogens with either antibodies or complement system components to enhance recognition by immune cells. Immunoglobulin G (IgG) antibodies binding to Fcγ receptors (FcγRs) trigger different functions: secretion of cytokines and chemokines, antibody-dependent cellular cytotoxicity (ADCC), phagocytosis and antigen presentation (182, 183). FcγRs, mainly expressed on APCs, are divided into three classes. The activating FcγRs are FcγRI (CD64), FcγRIIA/C (CD32A/C), and FcγRIIIA (CD16A). Pathogens are much more effectively engulfed by APC when opsonized (182-186) and are more efficiently presented to T cells (187, 188). At the start of the study, it was unknown if opsonization alters MR1-presentation and modifies MAIT cells responses. This prompted us to investigate if MAIT cells respond differently to IgG-opsonized bacteria compared to their non-opsonized counterparts.

4.1.1 MAIT cell functionality increases upon stimulation with IVIg-opsonized *E. coli*

As a model organism, we used *E. coli* D21, a common lab strain. Intravenous immunoglobulin G (IVIg) was used to opsonize *E. coli* (IVIg-*E. coli*). In the presence of THP-1 pulsed IVIg-*E. coli* stimulation, blood MAIT cell functions were increased with a superior production of IFNγ, TNF, IL-17A, GzmB and CD107a degranulation compared to non-opsonized *E. coli* (Figure 4A). This was in line with the recent observation of Banki et al (189), that MAIT cells increase IFNγ and TNF upon stimulation with IgG-opsonized *E. coli*. Furthermore, our data showed that MAIT cell responses to IVIg-*E. coli* were highly polyfunctional: the percentages of cells expressing GzmB+IFNγ+TNF+ or GzmB+IFNγ+ profiles were augmented (Figure 4B). This was reflected in the transcription factor profile with an increase in T-bet expression (Figure 4C and D), which is important for the Th1 phenotype in MAIT cells. The response of MAIT cells to IVIg-*E. coli* was more MR1-dependent than to non-opsonized *E. coli* (paper I, Fig. 1D left and Suppl. Fig. 1F), indicating that antigen presentation is probably more efficient in opsonized conditions.

To test if tissue MAIT cells respond similarly to opsonized pathogens, we used MAIT cells from tonsils, a readily accessible lymphoid tissue. MAIT cells from tonsils also increased IFNγ, IL-17A, GzmB secretion and CD107a degranulation upon IVIg-*E. coli* stimulation compared to non-opsonized *E. coli* (paper I, Suppl. Fig. 1 A and B). Of note, the level of IL-17A production was higher in tonsil than in blood, concordant with the previously observed higher IL-17A production in mucosal tissue (51, 57, 110).

The sensitivity of antigen detection is important during infection, and we therefore investigated the dose response of MAIT cells to IVIg-*E. coli* stimulation. At a suboptimal bacterial dose, opsonization enhanced the cytokine production of MAIT cells (paper I, Fig. 3A and Suppl. Fig. 2A). Furthermore, increased concentration of IVIg amplified this pattern. Thus, bacterial IgG opsonization reduces the sensitivity threshold of MAIT cells, allowing

detection of lower concentrations of bacteria. The kinetics of MAIT cell responses were also faster to stimulation with IVIg-*E. coli* than to non-opsonized *E. coli* (paper I, Fig. 3B and Suppl. Fig. 2B).

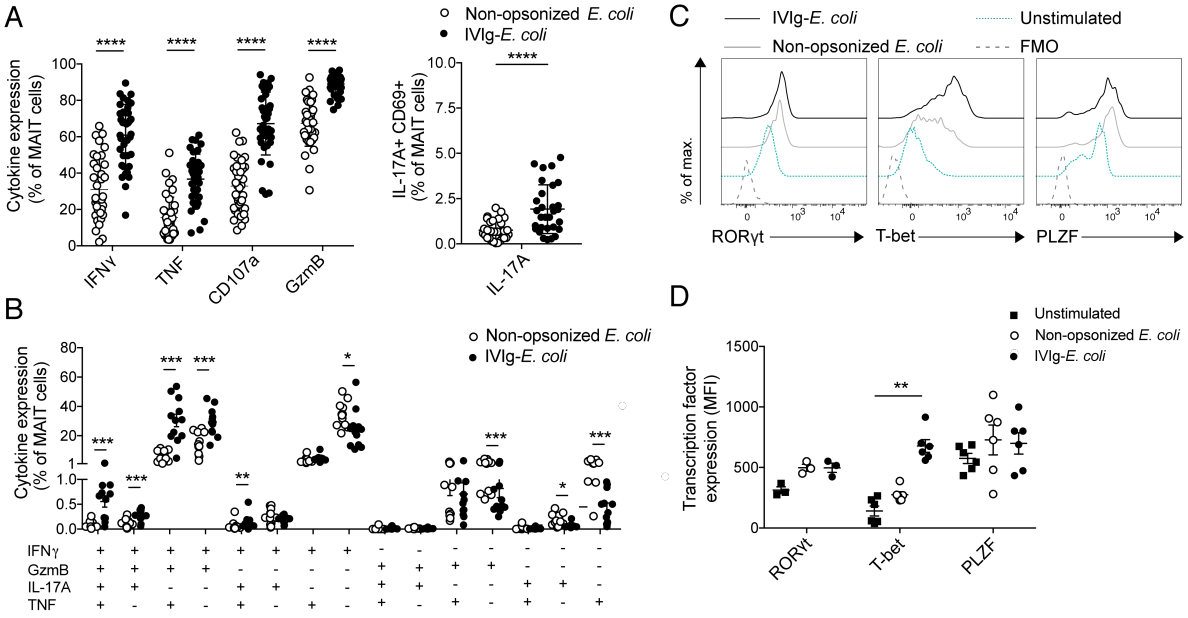

Figure 4. Opsonized bacteria enhance MAIT cell functionality. (A) Frequency of MAIT cells expressing IFNγ, TNF, CD107a, GzmB and IL-17A upon stimulation with IVIg-*E. coli* or non-opsonized *E. coli*. (B) Polyfunctional profile of MAIT cell cytokine production. Histogram plots (C) and combined data (D) of the expression of RORγT, T-bet and PLZF in MAIT cells upon stimulation. Adapted from paper I (190).

4.1.2 FcγR triggering increases MR1 antigen presentation to MAIT cell

MR1 surface expression on THP-1 cells increased in the presence of IVIg-*E. coli* (Figure 5A and B). As a mirror effect, TCR triggering in MAIT cells also increased upon IVIg-*E. coli* stimulation (paper I, Suppl. Fig. 1C and D), suggesting a more efficient antigen presentation to MAIT cells. Therefore, we aimed to understand if triggering of FcγR increases MR1 antigen presentation. We first blocked binding of IVIg-*E. coli* to the FcγR, using either Fc fragment from human IgG to saturate the FcγR expressed on the THP-1, or by deglycosylating the IVIg before opsonizing the bacteria. Indeed, glycosylation is essential to bind FcγR (191, 192). By blocking FcγR binding, the upregulation of MR1 surface expression in response to IVIg-*E. coli* was inhibited (Figure 5C), the extent of TCR downregulation on MAIT cells was reduced (paper I, Suppl. Fig. 3E) and cytokine response was back to the level of the non-opsonized condition (Figure 5D, E and F; paper I, Suppl. Fig. 3A and E). Taken together, these data demonstrate that FcγR triggering is essential to increase MR1 antigen presentation in this system.

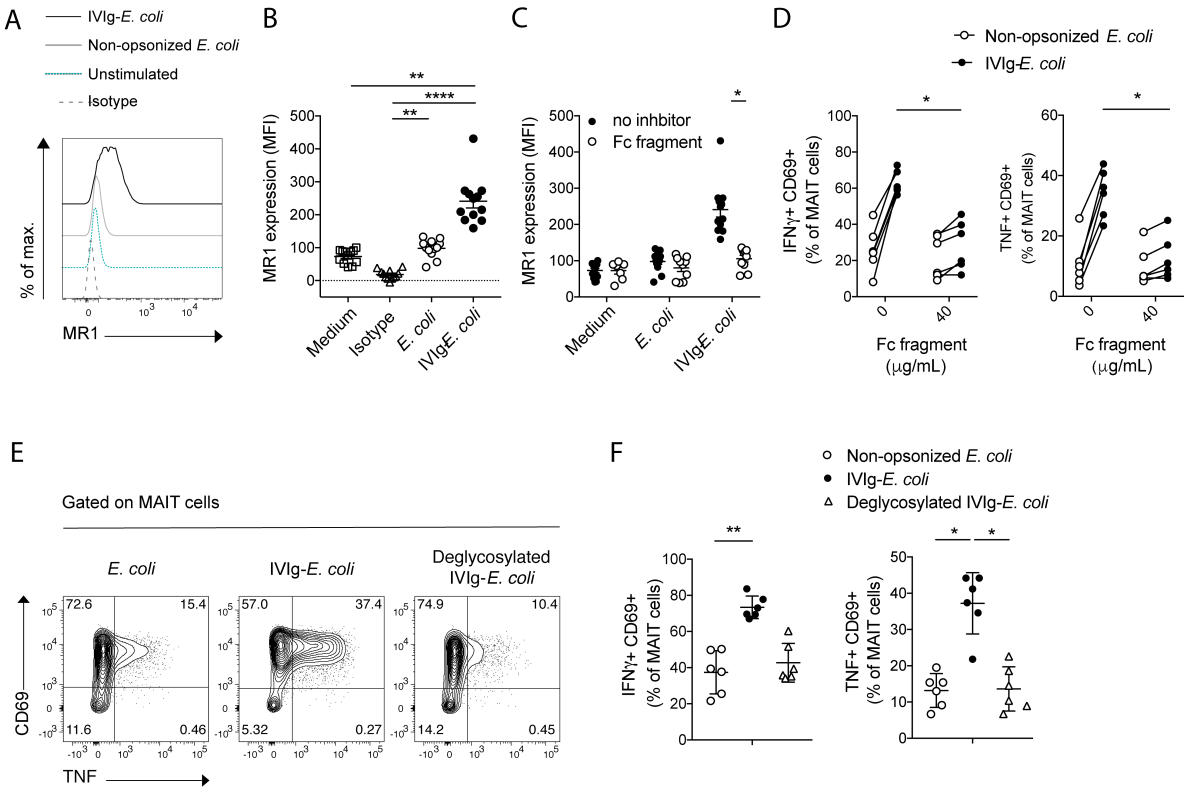

Figure 5. FcγR triggering increases MR1 antigen presentation to MAIT cells. Histogram (A) and combined data (B) of MR1 cell surface expression on THP-1 fed with IVIg-*E. coli* or non-opsonized *E. coli* for 3 h. (C) MR1 cell surface expression on THP-1 stimulated with IVIg-*E. coli* or non-opsonized *E. coli* in presence of Fc fragment for 3 h. (D) IFNγ+CD69+ and TNF+CD69+ expression in MAIT cells stimulated with IVIg-*E. coli* or non-opsonized *E. coli* with or without Fc fragment for 24 h. Flow cytometry plots (E) and combined data (F) of IFNγ+CD69+ and TNF+CD69+ in MAIT cells stimulated with deglycosylated IVIg-*E. coli*, IVIg-*E. coli* or non-opsonized *E. coli* for 24 h. Adapted from paper I (190).

To further dissect how FcγR triggering interacts with MR1 ligand loading, we interrogated different steps of the antigen presentation pathway using specific chemical inhibitors (Figure 6). First, we inhibited phagocytosis by using cytochalasin D, an actin polymerization inhibitor. Without phagocytosis, there is no bacterial processing and no antigen presentation. At steady state, MR1 is retained in the ER and travels to the cell surface only if bound to a ligand (38). Exposing THP-1 cells to brefeldin A, an ER egress inhibitor, abrogated MR1 cell surface expression in all conditions. MR1 may acquire new antigens during recycling, but for a new ligand to be bound, the previous ligand needs to be unloaded. This can happen in an acidic compartment since the Schiff base binding, essential for 5-OP-RU binding to MR1, is labile in acidic condition (44). Bafilomycin A, an inhibitor of lysosomal acidification, partially inhibits MR1 expression after IVIg-*E. coli*, suggesting that MR1 acquisition of new antigen in the endosome may occur in this condition. Finally, the inhibition of Syk using R406 inhibitor, that transmits signals from the FcγR, inhibits MR1 cell surface expression. Altogether, these findings suggest that phagocytosis, FcγR triggering, MR1 ER egress and passage in a lysosomal compartment are crucial for enhanced MR1 antigen presentation to MAIT cells.

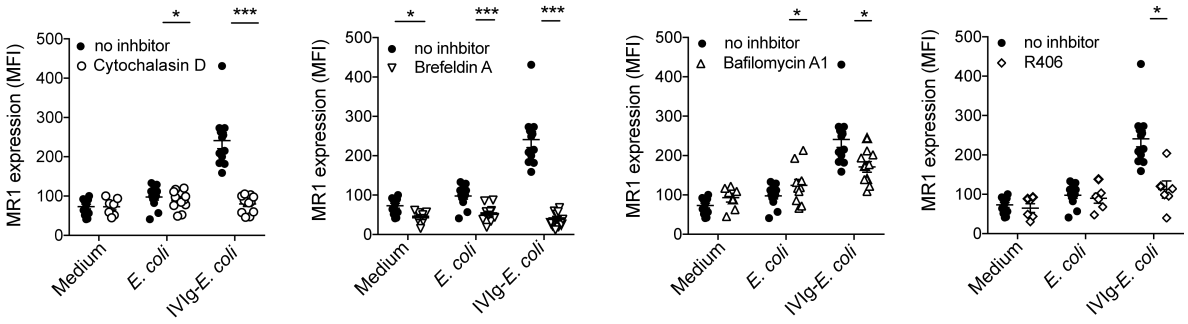

Figure 6. Increased MR1 antigen presentation is dependent of uptake, ER egress, lysosomal acidification and FcγR triggering. MR1 cell surface expression on THP-1 stimulated IVIg-*E. coli* or non-opsonized *E. coli* with or without chemical inhibitors for 3 h. Adapted from paper I (190).

4.1.3 Sera from vaccinated individuals boost MAIT cell function

S. pneumoniae is a Gram-positive pathogen that can colonize the upper respiratory tract, especially during childhood. It is an opportunistic pathogen, which can cause mild to severe diseases, including pneumonia, sepsis and meningitis (193), particularly as secondary infections (194) and in immunodeficient patients (195, 196). *S. pneumoniae* have many virulence factors, the major being the capsule (193). The humoral immune response raises antibodies directed against the capsular polysaccharides but also against protein antigens (197). Opsonization of *S. pneumoniae* with IgG allows more efficient processing by macrophages (197, 198). In addition, vaccination with the 13-valent *S. pneumoniae* vaccine (Prevnar-13) elicits an antibody response that prevents infection against the serotypes included in the vaccine (199-201). The sera from 9 healthy donors, taken before and after vaccination with the Prevnar-13 vaccine were characterized in a previous clinical trial for *S. pneumoniae* specific-antibody concentration and opsonophagocytic activity titer (199). We pooled the sera of these 9 donors to opsonize the vaccine strain *S. pneumoniae* 19A, and use it to stimulate MAIT cells and assess their functional profile. *S. pneumoniae* 19A opsonized with serum after vaccination elicited higher IFNγ and TNF secretion, and more CD107a degranulation in MAIT cells compared to *S. pneumoniae* 19A opsonized with serum before vaccination (Figure 7). The MAIT cell responses to *S. pneumoniae* 19A were MR1-dependent, as blocking MR1 with the Ac-6-FP reduced IFNγ and TNF production (paper I, Suppl. Fig. 4C). Altogether, these data suggest that the antibodies raised by the vaccination indirectly allow the augmentation of MAIT cell functions.

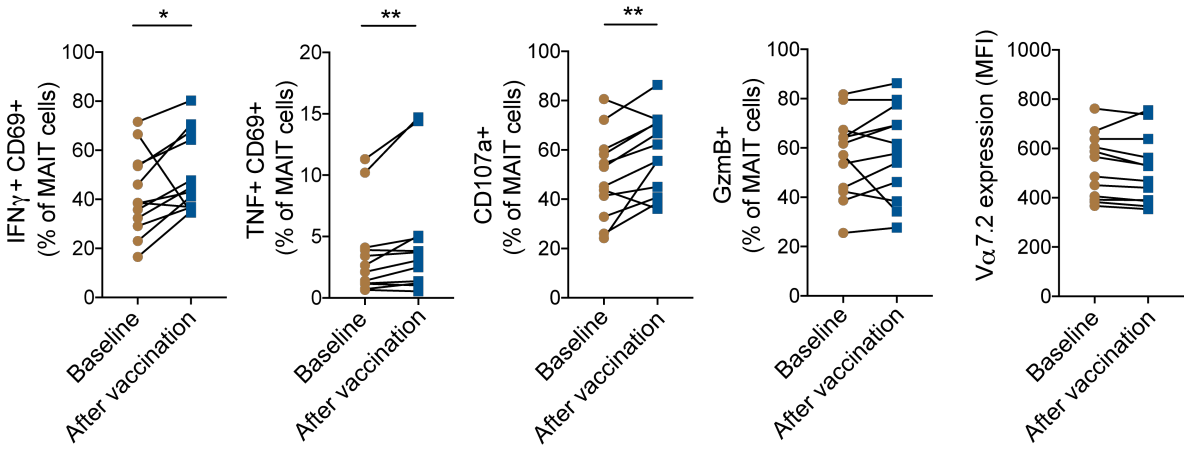

Figure 7. Percentage of MAIT cells expressing IFNγ+CD69+, TNF+CD69+, CD107a+, GzmB+ and Vα7.2 expression after a 24 h stimulation with THP-1 cells pulsed with *S. pneumoniae* opsonized with sera taken before, or after vaccination with the 13-valent pneumococcal vaccine. Adapted from paper I (190).

4.1.4 Implication for MAIT cells in the vaccine field

The role of MAIT cell in vaccine-induced immunity is currently unclear. Few published studies concerned the administration of the 5-OP-RU antigen as a vaccine. In mice, vaccination with the antigen 5-OP-RU combined with TLR9 agonist (123) or IL-23 (128) contributes to protection against *Legionella longbeachae* by increasing MAIT cell numbers in the lungs and allowing better bacterial load control. Moreover, the protection occurs earlier compared to non-vaccinated mice. However, another study using the combination of 5-OP-RU + TLR9 agonist as vaccine in the context of MTB infection did not see an improvement of bacterial load control (126).

A few other studies investigated MAIT cells after traditional vaccination. In the context of Bacillus Calmette-Guerin (BCG) vaccine administration in humans, MAIT cells were activated after *in vitro* stimulation (138), while they were directly activated *in vivo* in the case of macaques (202). A challenge study with live *S.* Typhi showed that MAIT cells are activated and decreased in the blood of volunteers that developed typhoid fever (Salerno, 2017). Live attenuated vaccine of *F. tularensis* in mice induces MAIT cell proliferation and cytokine production in the lung (124). Furthermore, MAIT cells perform a helper function by secreting GM-CSF that supports monocyte maturation and the subsequent recruitment of CD4+ T cells (129). Recent findings by the group of Paul Klenerman in Oxford show that MAIT cells are important for optimal immune responses to adenoviral vaccine vectors (203). In this context MAIT cells participate as innate viral sensors and amplify the innate response to the adenovirus vector with enhanced adaptive CD8+ T cell responses to the vaccine encoded antigen. These results open a new avenue in the study of MAIT cells in vaccinology.

Looking at the humoral side of vaccine immunity, MAIT cells may be mobilized at both sides of the response, during the initiation of B cell responses and during the effector phase of humoral immunity. Two animal studies show that MAIT cells are able to provide B cell help, through the maturation of naïve B cells and the induction of antibody production. The supernatant of MAIT cells from Simian immunodeficiency virus (SIV)-vaccinated macaques

stimulated with CD3/CD28 increased CD38 and CD69 expression on naïve B cells and was able to induce tissue-like memory B cells, which in macaques is the equivalent of plasma cells in humans. Furthermore, blood MAIT cell frequency correlated with the presence of some specific clones of memory B cells and with the titer of SIV-specific antibodies in rectum, suggesting that vaccination against SIV may trigger MAIT cells to help B cells maturation and secretion specific antibodies (141). Another study in mice in the context of *Vibrio cholerae* infection showed that the adoptive transfer of MAIT cells into mice lacking T cells promote pathogen specific IgA secretion upon challenge (62). Both studies indicated that MAIT cells helped initiate B cell responses.

In our study, we showed that IVIg-*E. coli* enhanced MAIT cell cytokine and cytotoxic protein production (Figure 8). The sensitivity to bacteria was also higher, so that MAIT cell responded to IVIg-*E. coli* at a lower bacterial dose. Furthermore, the kinetics of the response was also faster, suggesting that *in vivo* MAIT cells would respond quicker to opsonized microbes. The mechanism relies on activating FcγR. By triggering FcγR, IVIg-*E. coli* increase MR1 antigen presentation and antigen detection, leading to enhancement of MAIT cell function. Finally, we showed that the antibodies raised against *S. pneumoniae* during vaccination boosted MAIT cell cytokine production and degranulation. Altogether, we demonstrated that MAIT cells participate in the effector phase of humoral immunity.

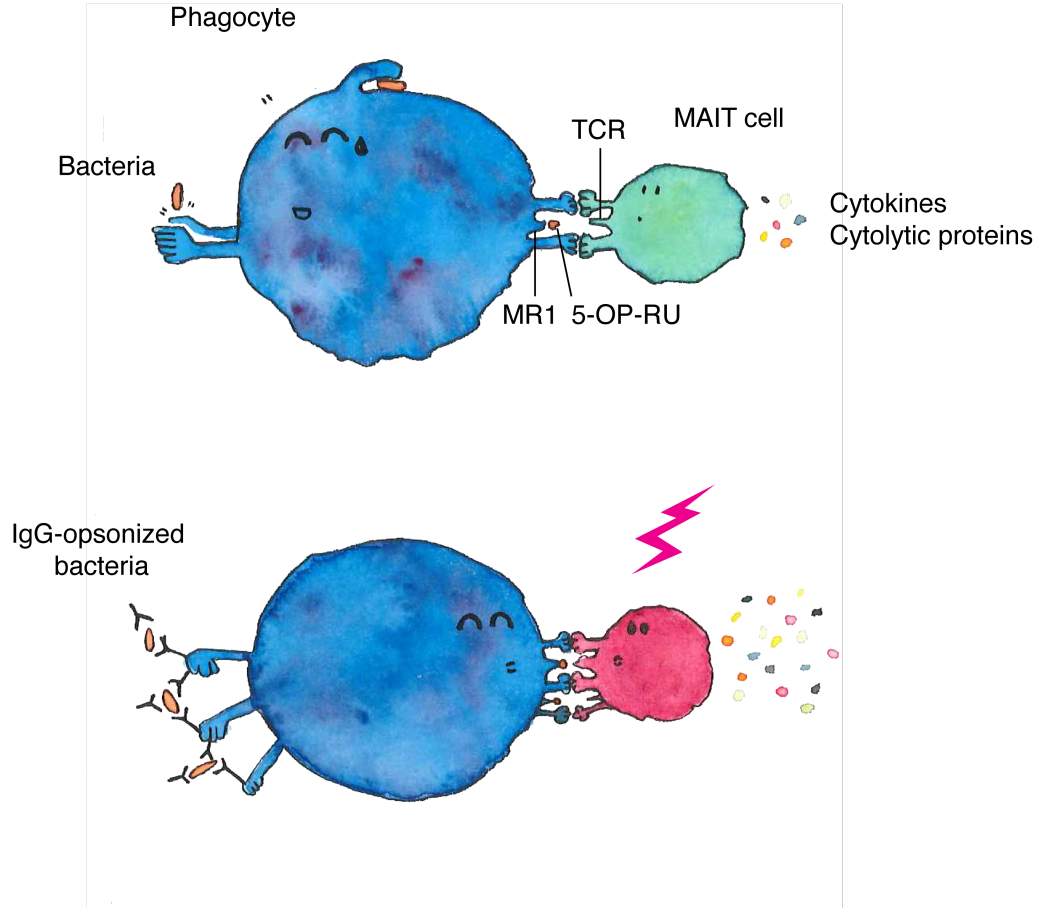

Figure 8. Illustration of MAIT cells responding to IgG-opsonized bacteria compared to non-opsonized one.

4.2 MAIT CELL CYTOTOXIC RESPONSES OVERCOME CARBAPENEM RESISTANCE IN *E. COLI*

After investigating MAIT cell responses against opsonized *E. coli*, we next examined MAIT cell-mediated antimicrobial activity against antibiotic-resistant *E. coli*. We focused our effort on CREC, which are highly drug-resistant organisms and constitute a global public health threat (163, 168). Due to the highly conserved nature of riboflavin synthesis and the critical role of riboflavin for bacterial metabolism, we hypothesized that MAIT cells are able to mediate antimicrobial activity even against bacteria that have developed antibiotic resistance.

4.2.1 Temporal expression of MAIT cell cytolytic proteins regulates bacterial load control *in vitro*

To assess MAIT cell antimicrobial activity against CREC, we initially developed a MAIT cell antimicrobial activity assay against drug-sensitive *E. coli* using *in vitro* expanded MAIT cells. Resting blood MAIT cells do not express cytolytic molecules, such as Gnly, GzmB, or perforin but do express GzmA (paper II, Fig. 1J and (82, 83)). MAIT cells cultured *in vitro* in the presence of 5-OP-RU antigen and the cytokines IL-2 and IL-7 upregulated their cytolytic granule content. The culture of cells not only prime MAIT cells for killing infected cells but also allowed us to have sufficient cell number to perform the killing assay. After a 15-day culture in the aforementioned conditions, nearly all MAIT cells expressed GzmB, GzmA, perforin, and Gnly (paper II, Fig. 1J). This increase in cytotoxic molecules reflected their killing capacities. Resting MAIT cells were not able to kill HeLa cells infected with the drug-sensitive *E. coli* EC120, while *in vitro* primed MAIT cells degranulated and killed HeLa cells (Figure 9A and B). Caspase 3 activation in the target cells was the read-out to identify apoptotic cells (Figure 9B). Prolonged cultures of MAIT cells were far more efficient in exerting MR1-dependent bacterial load control within the target cells compared to 2 days culture (Figure 9C and paper II, Fig. 1E), which coincided with the increased expression of Gnly (paper II, Fig. 1J)

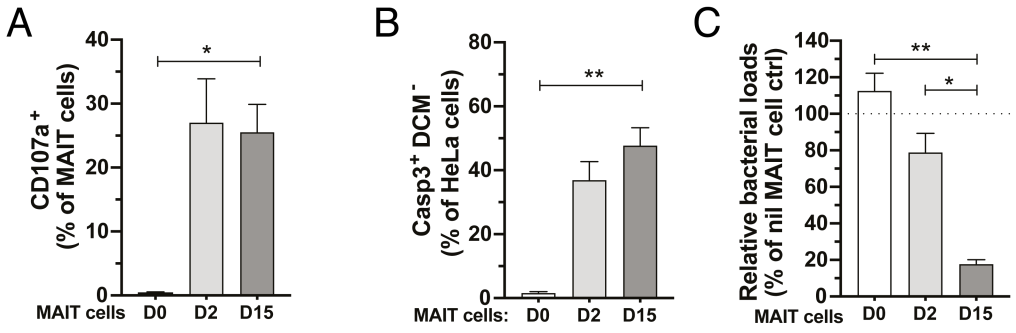

Figure 9. Temporal regulation of MAIT cell cytotoxic abilities. MAIT cell degranulation (A), apoptosis (B) and bacteria load (C) in HeLa cells after co-culture of EC120-infected HeLa cells with resting MAIT cells or MAIT cells after 2 or 15 days of culture. Adapted from paper II (204).

The killing of bacteria was not simply caused by apoptosis of the target cells (paper II, Fig. 2L), or disguised by the bacterial release into the extracellular environment (paper II, Fig. 1F), but by direct MAIT cell killing of the bacterial cells. GzmB, GzmA, perforin, and Gnly

were found within the HeLa cells, indicating that the cytotoxic proteins were delivered into the infected cells (paper II, Fig. 2A). Furthermore, with a fluorescent GzmB substrate, we detected active GzmB in the infected HeLa cells when co-cultured with MAIT cells (paper II, Fig. 2H), indicating that GzmB was delivered into the target cells. Lastly, the expression of GzmB and Gnly in MAIT cells correlated with the reduction of bacterial viability (paper II, Fig. 1D-G). To confirm the cell-associated bacteria killing ability were linked to MAIT cell cytotoxic capacity, we blocked the cytolytic machinery using pharmacological inhibitors. Inhibiting MAIT cell degranulation with the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) in the presence of Mg^{2+} abrogated MAIT cells degranulation, caspase 3 activation in the target cells, and cell-associated bacterial load control (paper II, Fig. 1J-L). Moreover, blocking GzmB with N-acetyl-L-isoleucyl-L-α-glutamyl-N-[(1S)-2-carboxy-1-formylethyl]-L-threoninamide trifluoroacetate (Ac-IETD-CHO) affected the bacterial load control and impaired killing of the target cell, but did not affect MAIT cell degranulation. By contrast, blocking GzmA, IFNγ, TNF, and IL-17A did not affect MAIT cell cytotoxic functions (paper II, Fig. 1J-L).

Since bacterial control is a crucial event in mucosal tissues, we tested if tissue MAIT cells have similar cytolytic capabilities using nasopharyngeal (NP) samples from healthy individuals. NP MAIT cells at steady-state expressed low levels of cytotoxic molecules, similar to blood MAIT cells. Culture of the cells in IL-7, IL-2, and 5-OP-RU upregulated GzmB, Gnly, perforin, and GzmA at a rate similar to that observed in matched blood MAIT cells, but at a somewhat lower magnitude (paper II, Fig. 1K and L). Nevertheless, NP MAIT cells were efficient at killing HeLa cells infected with EC120 through cytolytic protein-dependent pathway (paper II, Fig. 1M).

Altogether, these data demonstrated that MAIT cells kill infected cells and cell-associated bacteria through exocytosis of cytotoxic molecules. Moreover, the antimicrobial properties of MAIT cells are maintained in the mucosa.

4.2.2 Antimicrobial properties of MAIT cells are maintained against carbapenem-resistant *E. coli* (CREC)

4.2.2.1 MAIT cells are activated by CREC and kill CREC-infected cells

Next, we assessed if the cytotoxic properties of MAIT cells were maintained against CREC clinical isolates. We first confirmed that CREC strains EC234, EC241, EC362 and EC385 expressed the riboflavin pathway. All the CREC strains grew in a riboflavin-free medium and expressed the *ribA* gene coding for the first enzyme in the riboflavin pathway (paper II, Suppl. Fig. 3A-I). Stimulation of the PBMC pool with CREC strains EC234, EC241, EC362, and EC385 induced MAIT cell cytokines production and degranulation in an MR1-dependent way (paper II, Fig. 3A and B). To determine if MAIT cells can kill CREC-infected cells, we co-culture *in vitro* primed MAIT cells with CREC infected-HeLa cells and assessed caspase 3 activation and bacterial count in the target cells. MAIT cells retained their MR1-dependent

killing capacities against CREC-infected HeLa cells and could also reduce the cell-associated bacteria viability, in a TCR-dependent way (Figure 10A and B and paper II, Fig. 3C and D).

4.2.2.2 Cytolytic proteins produced by MAIT cells damage free-living CREC

Since MAIT cell cytolytic granule content was found in the surrounding environment (paper II, Suppl. Fig. 4A-C), we explored the capacity of MAIT cells to kill extracellular bacteria. The CREC strains EC234 and EC362 were incubated in supernatants from the co-culture of 5-OP-RU-pulsed 293T-hMR1 cells with MAIT cells. Bacterial damage was assessed using SYTOX Green, an impermeable nucleic acid dye that will stain bacteria only if there is cell membrane damage. In the presence of MAIT cell supernatants, SYTOX Green staining increased in the CREC strains, suggesting bacterial membrane damage (Figure 10C and D). Control supernatants from 293T-hMR1 cells pulsed with 5-OP-RU did not affect the bacteria. Bacterial viability was also reduced in presence of MAIT cells supernatant but not totally suppressed (paper II, Fig. 4D). Depletion of Gnly from the supernatants decreased SYTOX Green staining, indicating that the supernatants partially lost the antimicrobial properties due to Gnly removal (paper II, Suppl. Fig. 6K and L). Gnly is a pore-forming protein targeting the bacterial membrane (114), and without Gnly the membrane damage was alleviated. We then examined if the cytotoxic molecules could be present inside the CREC. Gnly was found inside CREC EC234 and EC362 (paper II, Fig. 5G and H), while GzmB was mostly detected in presence of Gnly. Perforin and GzmA were not detected inside supernatant-treated bacterial cells (paper II, Suppl. Fig. 7A). Interestingly, GzmB and Gnly were detected mainly in SYTOX Green positive EC234 and EC362 bacteria (Figure 10E and F), indicating that GzmB and Gnly participate in disrupting bacterial membrane integrity and facilitating bacterial killing.

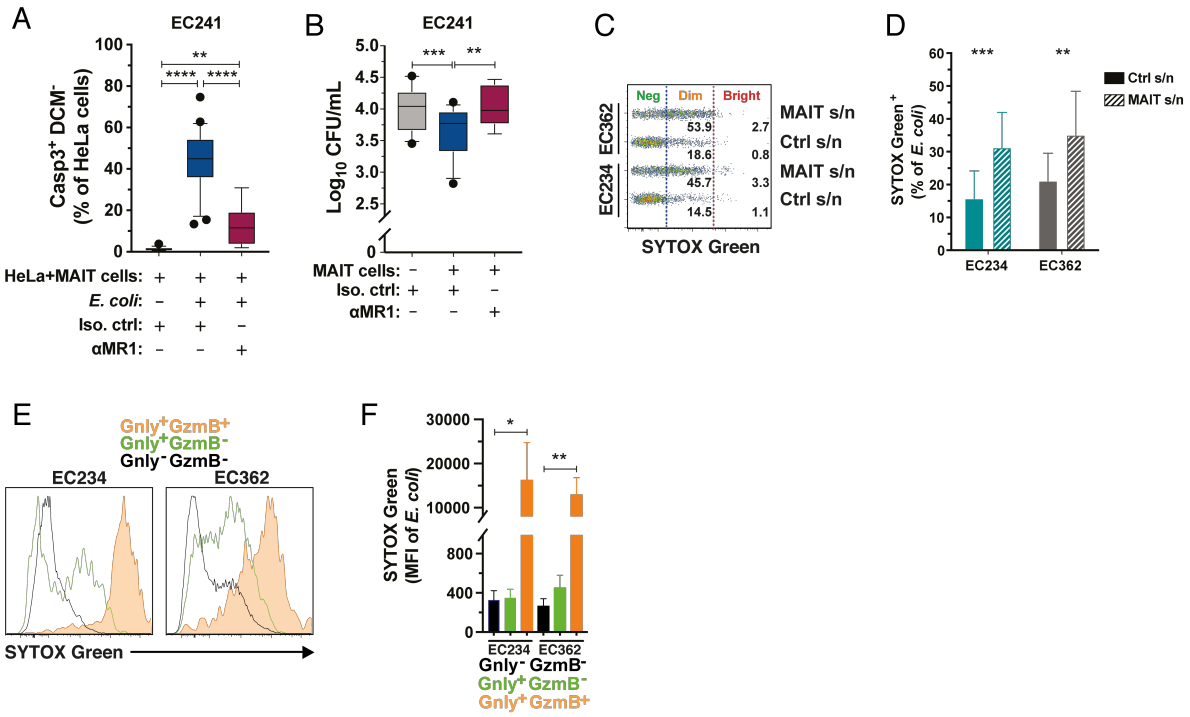

Figure 10. MAIT cell antimicrobial activity against CREC. Caspase 3 activation (A) and bacterial load (B) in *E. coli* EC241-infected HeLa cells after co-culture with MAIT cells with or without anti-MR1 antibody. Representative plot (C) or combined data (D) of *E. coli* EC234 and EC362 SYTOX Green staining after incubation with MAIT cell supernatants or control supernatant. Representative histogram (E) and expression (F) of SYTOX Green with GzmB and Gnly in *E. coli* EC234 and EC362 after incubation with MAIT cells supernatants. Adapted from paper II (204).

Overall, these data demonstrate that MAIT cells were activated by CREC and mediated cytotoxicity against CREC-infected HeLa cells by inducing HeLa cell death and by controlling cell-associated bacterial loads. Furthermore, the cytolytic proteins Gnly and GzmB produced by MAIT cells damaged bacterial membrane and participated in the killing of extracellular CREC.

4.2.3 MAIT cell cytolytic proteins synergize with carbapenem to enhance killing of extracellular CREC

Since MAIT cell granule contents induce bacterial membrane damage, we investigated if MAIT cell supernatants were able to enhance carbapenem antibiotic activity against CREC strains. To that end, we incubated CREC EC234 and EC362 with MAIT cell supernatants in the presence of the carbapenem antibiotics imipenem, ertapenem, or meropenem. Bacterial membrane damaged was greatly enhanced by the combination of MAIT cell supernatants and carbapenems, compared to the supernatants alone (Figure 11A and B; paper II, Suppl. Fig. 4I), suggesting that GzmB and Gnly greatly enhanced the antibiotic properties of carbapenems against even highly carbapenem-resistant *E. coli* strains.

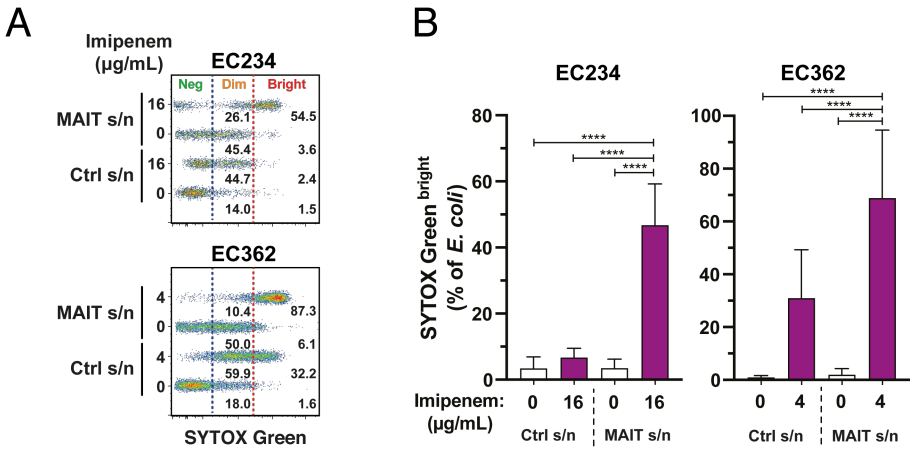

Figure 11. Representative FACS plots (A) and combined data (B) of SYTOX Green staining in *E. coli* EC234 and EC362 after incubation with MAIT cell supernatants or control supernatant in presence of imipenem for 2 h. Adapted from paper II (204).

Moreover, the growth of CREC EC234 and EC362 was slowed down or suppressed in the presence of MAIT cell supernatants combined with imipenem, at imipenem concentration below the minimum inhibitory concentration (MIC) (paper II, Suppl. Fig. 5A and B). Of note, EC234 has a MIC to imipenem >32 µg/mL, while the XDR strain EC362 has a MIC to imipenem of 8 µg/mL (paper II, Table S1). Thus, MAIT cell supernatants decreased the imipenem concentration needed to inhibit the growth of those strains. To further characterize

this effect, we performed a kinetics study to assess bacterial killing by MAIT cell supernatants in combination with imipenem over time. Over the course of 24 hours, the effect of MAIT cell supernatant with titrated amounts of imipenem was assessed on the bacterial viability of CREC EC234 and EC362 strains. MAIT cell supernatants combined with imipenem kill the bacteria at an imipenem concentration below the MICs of those strains (paper II, Fig. 4G and H). Interestingly, the amount of GzmB and Gnly present in the MAIT cell supernatants were higher in the donors that abrogated bacterial growth (paper II, Fig. 5A and B). Moreover, the amount of GzmB and Gnly in the supernatants correlated with the inhibition of bacterial growth (paper II, Fig. 5C-F). No difference was noted in perforin and GzmA levels (paper II, Fig. 5A and B). It is also notable to highlight that in some experiments, the CREC strains regrew in the presence of combined imipenem and MAIT cell supernatants, probably due to the finite amounts of cytolytic proteins in the MAIT cell supernatants in these cultures.

Altogether, these results suggest that MAIT cell cytolytic proteins, particularly Gnly and GzmB synergize with carbapenem to enhance and restore the bactericidal activity of carbapenems.

4.2.4 Conclusions

In this paper, we explored the mechanism underlying MAIT cell antimicrobial cytotoxicity. First, we showed that MAIT cells' direct killing of cell-associated bacteria occured in an MR1-dependent fashion. It is interesting to note that these antimicrobial properties are temporally regulated since resting MAIT cells do not have killing capacities. Then, we confirmed that these antimicrobial properties extended to CREC. The cytolytic proteins produced by MAIT cells were present in the surrounding environment and the secreted molecules damaged the membrane of free-living bacteria, hence reducing viability. These cytolytic proteins also increased the membrane permeability of CREC strains, hence restoring the bactericidal effect of carbapenem antibiotics. This effect was primarily mediated by GzmB and Gnly. Gnly is an antimicrobial protein that forms pores on bacterial membrane (114). GzmB exerts important antimicrobial functions by cleaving vital proteins and antioxidant defense enzymes that ultimately lead to bacterial cell death (113). Gnly and GzmB were both detected in the damaged bacteria incubated in MAIT cell supernatants, but GzmB was only detected in the presence of Gnly, suggesting that Gnly is needed to allow GzmB access into the bacterial cells. The addition of carbapenems further exacerbated CREC bacterial damage, suggesting the restoration of carbapenems bactericidal activity. Enhanced penetration of carbapenems into the bacteria was a likely explanation of increased bacterial death following co-incubation with MAIT cell supernatants, and this may be due to the increased membrane damage caused primarily by Gnly, and to a lesser extent by GzmB. Thus, MAIT cell cytolytic proteins may overcome the impermeability mechanism of drug resistance. Overall, these data highlight the important antimicrobial effector functions exhibited by MAIT cells (Figure 3).

4.3 *S. AUREUS* EVADES MAIT CELL RECOGNITION WITH THE HELP OF LUKED TOXIN

Finally, we explored if microbes may directly target MAIT cells to avoid recognition and circumvent the rapid immune response by these cells. Mechanisms of evasion from MAIT cell recognition have been proposed in two studies, but both are related to the MR1 antigen presentation pathway. The study by Preciado-Llanes *et al* (132) showed that modification of the *ribB* gene encoding the enzyme involved in the riboflavin secretion pathway in bacteria can block MAIT cell activation. Another study demonstrated impairment of the MR1 antigen presentation due to viral infection (205). To assess the existence of other immune evasion mechanisms, we switched bacterial model and used *S. aureus*, for the reasons described below.

S. aureus is a gram-positive opportunistic pathogen that colonizes human skin and nose (206). The combined events of barrier breach and secretion of virulence factors can lead to pathogenesis and serious diseases such as sepsis, endocarditis, and pneumonia (207-209). Virulence factors secreted by *S. aureus* are key in the pathogenesis and carry redundant functions. They are composed of superantigens, cytolytic peptides, and pore-forming toxins. The *S. aureus* superantigen staphylococcal enterotoxin B (SEB) activates MAIT cells and renders them anergic to further bacterial stimulation (210). The pore-forming toxin leukotoxin ED (LukED) is part of the leukocidin family and is composed of two subunits: LukE and LukD. LukE first binds to the receptor before recruiting LukD. Then, the complex oligomerizes and inserts into the cell membrane to create a pore, ultimately leading to osmolysis (211). LukED binds to several chemokine receptors, including CCR5, CXCR1, and CXCR2 (212, 213) and to the duffy antigen receptor for chemokines (DARC) (214, 215). These receptors are widely expressed on immune cells such as T cells, NK cells, DC, and epithelial cells; therefore, LukED protects *S. aureus* from numerous types of effector cells. LukED is an important virulence factor for disease pathogenesis and mortality of the host (212-215).

From the literature, we know that *S. aureus* activates MAIT cells (27, 55). MAIT cells increase in blood and tonsils during *S. aureus* tonsillitis (216), but decrease in blood during *S. aureus* bacteremia (217). Since MAIT cells express high levels of CCR5, we were interested to investigate if LukED can also target MAIT cells.

4.3.1 LukED wipes out MAIT cells from a mixed culture

To investigate the effects of LukED on human MAIT cells, we incubated PBMC with recombinant LukED toxin and assessed the alterations of the MAIT cell population using flow cytometry. We first used the uniform manifold approximation and projection (UMAP) analysis (218), an unsupervised approach to analyze effects on the total lymphocyte population (paper III, Fig. 1A and B). In the LukED-treated conditions, the disappearance of the population marked by the projection of the 5-OP-RU-hMR1 tetramer staining was striking and suggested that LukED depletes the MAIT cell population. The effect of LukED on depleting MAIT cells was confirmed by traditional gating, and was almost complete in

absolute count or when calculated as percentage out of T cells (Figure 12A). In contrast, CCR5+ conventional non-MAIT T cells were not affected to the same extent as MAIT cells, in line with the lower CCR5 expression on CCR5+ conventional T cells compared to MAIT cells (Figure 12B and C and paper III, Suppl. Fig. 1D). The MAIT cell population was rescued by the addition of Maraviroc (MVC) to the culture, a CCR5 antagonist used in HIV therapy (paper III, Fig. 2A). The protective effect of MVC was weaker for the CCR5+ conventional T cells (paper III, Fig. 2C). It is interesting to note that LukED also targets effector memory T cells (TEM), terminally differentiated effector memory CD45RA+ T cells (TEMRA), and CD8+CD56+ conventional T cells through CCR5 or CXCR1, although the depletion of these subsets was less pronounced than MAIT cells (paper III, Fig. 1J-L and Suppl. Fig. 1H-L).

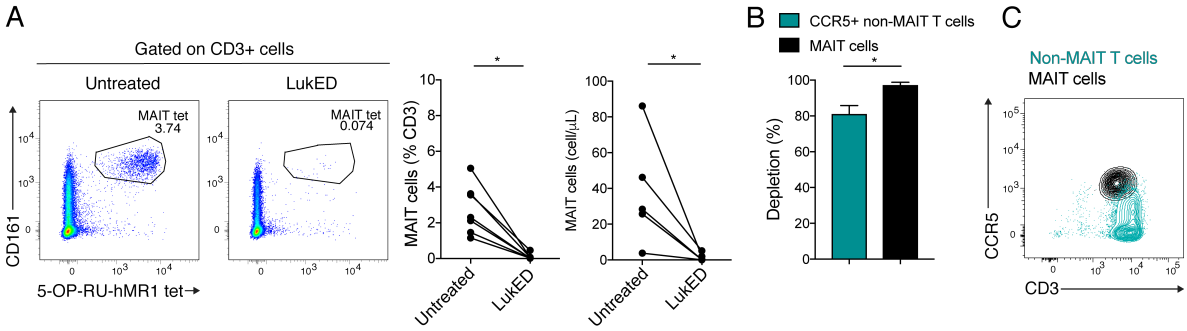

Figure 12. MAIT cells are more sensitive to LukED than CCR5+ conventional T cells. (A) Representative flow cytometry plots and combined data of the percentage and absolute count of MAIT cells upon LukED exposure. (B) Depletion rate of MAIT cells versus CCR5+ conventional T cells. (C) Representative flow cytometry plot of CCR5 expression in MAIT cells and CCR5+ conventional T cells.

LukED-mediated depletion of MAIT and CCR5+ conventional T cells occurred in a dose-dependent manner. However, MAIT cells were more sensitive than CCR5+ conventional T cells, since the dose needed to kill half of the population (50% of minimum inhibitory concentration or IC_{50}) was lower for MAIT cells (paper III, Fig. 2D). The length of exposure to the toxin did not have a major impact on toxicity, suggesting that the effect is rapid and that the toxin dose is the key paramater for LukED cytotoxicity (paper III, Fig. 2E).

To further confirm that LukED targets MAIT cells, we incubated PBMC with the supernatants of cultures of clinical strains that have been previously characterized for the absence or presence of LukED secretion (171). In presence of the supernatant from LukED-secreting strains, the percentage of MAIT cells declined while MAIT cells population exposed to LukED-free supernatant remained unchanged (paper III, Fig. 1M). Overall, these data demonstrate that LukED preferentially targets and depletes MAIT cells.

4.3.2 IL-12 and IL-18 activation may prevent LukED killing of MAIT cells

MAIT cells can be activated by stimulation with cytokines produced by myeloid cells in the innate response, such as IL-18 and IL-12. We therefore investigated how this mode of activation may impact the sensitivity of MAIT cells to LukED. PBMC were pre-stimulated with IL-12 and IL-8 for 20 hours before adding the recombinant LukED toxin for the last 2

hours of the assay. Interestingly, the pre-stimulation with cytokines partially protected MAIT cells from LukED; probably via the lowering of CCR5 levels on MAIT cells (paper III, Fig. 3A-C).

4.3.3 Sub-lethal LukED doses do not affect MAIT cell responses

Next, we explored if sub-lethal doses of LukED could impair MAIT cell activation, possibly due to signaling defects. MAIT cells were pre-incubated with sub-lethal doses of LukED before the addition of THP-1 cells pulsed with mildly fixed *S. aureus* strains either expressing LukED or not. The MAIT cell cytokine expression in response to stimulation was similar with or without sub-lethal LukED pre-treatment, irrespective of the bacterial strain (paper III, Fig. 3D and E). Altogether, these observations support the notion that that exposure to LukED levels below the toxic dose does not impair MAIT cell activation.

4.3.4 Can MAIT cells mediate antimicrobial function against *S. aureus* infected cells?

Since *S. aureus* can activate MAIT cells, we next aimed to investigate if MAIT cell could kill *S. aureus* infected cells and control bacterial loads. We used the protocol previously developed in paper II for that purpose. In this assay, gentamicin, an aminoglycoside antibiotic, was used to eliminate the remaining extracellular bacteria. Unfortunately, the *S. aureus* strains used were methicillin resistant *S. aureus* (MRSA) (β-lactam family) and resistant also to aminoglycosides, and thus no antibiotic from those classes could be used in the assay. We explored other options, but the choice was limited, as we needed a hydrophilic antibiotic that stay outside the cells. We turned our attention to the lysostaphin enzyme that cleaves glycine in the peptidoglycan layer and lyse the bacteria. Despite that lysostaphin should theoretically stay outside the cells (219), it unexpectedly also killed intracellular bacteria in our assay. Thus, we unfortunately had to abandon this experimental approach.

4.3.5 LukED targets mature NK cells

In the UMAP plot, the area corresponding to the CD56 projection was altered upon LukED exposure. This population corresponds to NK cells, and this inspired us to explore more in detail this cell type (paper III, Fig. 1A and B). Previously, it was described that LukED targets NK cells in CXCR1-dependent manner (213), but the different NK cell subsets were not investigated. NK cells are going through different stages of maturation and those can be identified using combinations of markers. Upon maturation, NK cells downregulate CD56, going from a $CD56^{bright}$ to $CD56^{dim}$ phenotype. Furthermore, mature cytotoxic NK cells gain KIR and CD57, and downregulate NKG2A. The FcγRIIIA receptor (CD16) is mainly expressed on $CD56^{dim}$ NK cells (220). NK cells may be important for immune control of *S. aureus* infection *in vivo* (221). Here, we studied NK cells in the same experimental setup as MAIT cells: PBMC were incubated in the presence of LukED toxin, and flow cytometry was subsequently used to evaluate the effect on NK cells.

Our data indicates that LukED depleted NK cells, however the depletion was not complete and $CD56^{dim}$ NK cells were particularly vulnerable while $CD56^{bright}$ NK cells were less so (paper III, Fig. 4A and B). This was probably due to the higher expression of CXCR1 and CXCR2 in $CD56^{dim}$ NK cells (paper III, Fig. 4C and D). Within the $CD56^{dim}$ NK cell population, NK cells expressing CD16, CD57, KIRD2L1, perforin or co-expressing CD57 and KIRD2L1 were more severely depleted (paper III, Fig. 4E and F). This phenotype corresponds to the more mature and cytotoxic NK cells. Thus, the LukED toxin targets mature effector NK cells.

4.3.6 Conclusions

S. aureus infections are widespread in hospital and community settings and are often methicillin resistant (MRSA). MRSA are becoming more and more resistant to antibiotics, thus reducing treatment options. Moreover, MRSA is responsible for numerous deaths (208, 222). An effective vaccine would help protect against infections, but vaccine design against *S. aureus* has been difficult due to the redundancy of the multiple virulence factors and lack of appropriate animal models (223). Indeed, the toxins do not act the same way in different species (224). Mobilization of MAIT cells by vaccination could be attractive due to their secretion of IL-17 and IL-22, and their localization in blood and skin, the sites of *S. aureus* infection (223).

In this paper, we explore how the toxin LukED affects MAIT cells in comparison to conventional T cells. We found that MAIT cells are hypersensitive to LukED. However, MAIT cells were rescued with the CCR5 antagonist MVC. Within the T cell pool, MAIT cells are the most sensitive T cell subset: CCR5+ and CD8+CD56+ conventional T cells, TEM, TEMRA cell were not as severely affected. Some of those conventional T cells may be part of the Th17 immunity, which is important in the defense against *S. aureus*. Indeed, primary immunodeficiency patients lacking Th17 cells due to genetic mutations are more susceptible to *S. aureus* (225). In this study we also unveil that the most mature and cytotoxic NK cell subsets are targeted and depleted by LukED.

MAIT cell TCR-dependent functions were maintained in presence of sub-lethal levels of LukED, indicating that MAIT cells may mediate effective immune defense when the bacteria do not produce high level of toxins. Interestingly, pre-activation with IL-12 and IL-18 protects MAIT cells against LukED probably as a consequence of the downregulation of CCR5 in response to stimulation. Antibody-based therapies directed against *S. aureus* toxins are in development to fight against *S. aureus* infection (226-228). This strategy has several advantages, including the protection and possible reconstitution of the pool of cells responsible for antibacterial effector functions. Given the fact that MAIT cells are particularly well equipped to fight bacterial infection, protecting these cells from depletion is most likely highly beneficial.

5 CONCLUDING REMARKS AND PERSPECTIVE

MAIT cells were first described 18 years ago (67) and the knowledge gathered in the last decade has considerably strengthened the field. MAIT cells are primarily antibacterial immune cells, due to the recognition of bacterial antigen but also act as innate sensors of viral infection through their cytokine-dependent activation mode. Thus, MAIT cells are broadly involved in immunity against infectious disease. Despite the knowledge we have about their cytokine response profile and cytolytic protein release, their functional role is not completely resolved yet. In this thesis work, we investigated the function of MAIT cells in different disease settings.

The MAIT cell response to bacteria changes if the bacteria are opsonized by IgG (paper I): their functional response is stronger and more polyfunctional. MAIT cells respond to lower bacterial dose if the bacteria are opsonized, showing increased sensitivity. Furthermore, MAIT cells respond faster to opsonized bacteria. The change in functional response is linked to FcγR triggering on APCs by the IgG-opsonized bacteria, which leads to increased MR1 antigen presentation. The relevance of the boost of functionality was validated using *S. pneumoniae* specific-IgG raised during vaccination. After vaccination with Prevnar-13 (vaccine containing 13 different strains of *S. pneumoniae*), the sera of vaccinated individuals had increased serotype specific antibodies (199). Using pooled sera taken before and after vaccination, the vaccine strain *S. pneumoniae* 19A was opsonized and used to stimulate MAIT cells. The MAIT cell response to *S. pneumoniae* opsonized with serum after vaccination displayed increased levels of IFNγ, TNF, and CD107a (degranulation) compared to *S. pneumoniae* opsonized with sera sampled before vaccination. Overall, these findings indicate that MAIT cells may constitute an effector arm of humoral immunity, more efficiently targeting bacteria that are also targeted by antibodies. One limitation of this study is that we did not have paired blood and serum from the *S. pneumoniae* vaccinated individuals. It would be interesting to repeat this experiment using such paired blood and serum samples. Since Prevnar-13 is a polysaccharide vaccine, it would be interesting to extend the study to a live attenuated vaccine such as BCG or the *S.* Typhi vaccine (Ty21a). Salerno *et al*. (102) assessed the phenotype of human MAIT cells for a month after *S.* Typhi challenge. MAIT cells declined in blood and were more activated in individuals that developed typhoid fever compared to those who did not develop disease. No *ex vivo* stimulation was performed in this study. Since *Salmonella* IgG-specific antibodies are important for increased phagocytosis during secondary challenge (229), it would be interesting to investigate the MAIT cell response in this context. Furthermore, MAIT cells probably have B cell helper function (62, 141, 230), and it could be interesting to investigate if B cell antibody production in the vaccinated individuals correlates with MAIT cell activation and possible migration to tissue.

Apart from B cell help, MAIT cells indirectly orchestrate other downstream antimicrobial responses through cytokine release (116). MAIT cells also have direct cytotoxic ability and kill bacterially infected cells (83, 103). We showed in paper II that MAIT cell cytotoxicity is

active also against cell-associated bacteria, including CREC, and is mediated by cytolytic proteins GzmB, perforin, and Gnly. The control of the cell-associated bacterial load by MAIT cells was dependent on the timing. MAIT cells seemed to need extended stimulation with IL-2, IL-7 cytokines to upregulate Gnly. It would be very interesting to know if this temporal regulation of MAIT cell killing ability also occurs *in vivo*. For instance, this could be done after 5-OP-RU vaccination in mice with transgenic expression of Gnly (114, 115) since rodents are devoid of Gnly. If MAIT cells need a long priming to become cytolytic against bacteria, this leaves a long time for the bacteria to cause disease. The cytotoxic proteins GzmB and Gnly released by MAIT cells in the surrounding environment damaged the membrane of free-living CREC. Interestingly, the addition of carbapenem to this environment enhanced the bactericidal effect of MAIT cells *in vitro*, showing that carbapenem synergizes with GzmB and Gnly. We speculated that the increased membrane permeability caused by GzmB and Gnly allow the penetration of carbapenem inside the bacteria, thus overcoming the impermeability mechanism of antibiotic resistance. To demonstrate that carbapenems penetrate the intracellular bacteria in the presence of GzmB and Gnly, one may use a fluorescently labelled carbapenem antibiotic (231) that could be detected by flow cytometry. It would also be interesting to test if MAIT cells could disarm other mechanisms of resistance, such as β-lactamases. Another aspect to test is if MAIT cell cytolytic ability extends to other resistant microorganisms. Yet another facet to investigate is the possible development of bacterial resistance to GzmB or Gnly. Indeed, mechanisms of evasion of MAIT cell recognition were recently shown to exist (132).

Along these lines, we have investigated further the ability of microbes to escape MAIT cell recognition. Some *S. aureus* strains produce SEB that render MAIT cells anergic to further stimulation (210). *S. aureus* secrete other virulence factors, including the pore-forming toxin LukED. In paper III, we found that MAIT cells are extremely sensitive to the LukED toxin. LukED also partially depleted TEM, TEMRA, and $CD56^{dim}$ NK cells but to a lower extent than MAIT cells, indicating that within the T cell pool MAIT cells are the most LukED-sensitive population. One may wonder if LukED is one of the causes of MAIT cell depletion during *S. aureus* bacteremia (217). Furthermore, we observed that clinical isolates known to secrete LukED depleted MAIT cells *in vitro*. Given the fact that *S. aureus* secrete many virulence factors, it would be interesting to use congenic strains lacking LukED to ascertain that the depletion is LukED-dependent. Unfortunately, this modification is very difficult to engineer in clinical bacterial strains and we were not able to pursue this idea for this study. It is also unknown what concentration of LukED is secreted at the site of infection. We showed that a low dose of toxin does not affect MAIT cell activation in response to antigen. On the other hand, IL-12 and IL-18 stimulation seems to partially protect MAIT cells from LukED, probably due to CCR5 downregulation. It would be interesting to determine the CCR5 expression on MAIT cells *in situ* during *S. aureus* infection, and if the downregulation protects MAIT cells *in vivo*. From the literature, we know that CCR5 expression is increased in blood MAIT cells during acute HIV infection (155), while it is decreased in chronic HCV

(232). In tissue, CCR5 was found to be expressed on healthy and diseased liver MAIT cells (108), and increased on peritoneal MAIT cells in decompensated liver cirrhosis (233).

MAIT cells are present in many tissues, and their functions may differ between locations. In mucosal tissues, MAIT cells seem to lean towards a more pronounced Th17 profile. At least in the nasopharynx, the MAIT cell cytolytic functions seem to be similar to blood. Many aspects remain to be unveiled, in particular if tissue localization directs the functionality. If MAIT cells traffic between tissues, do the functions adjust to the local environment? It was recently found that MAIT cells can have tissue repair functions, but it is also possible that some functions are yet to be uncovered. For instance, MAIT cells seem to have B cell helper function. To what extent is that compatible or competitive with Tfh cells? MAIT cells also produce IL-10 under some circumstances. Does this mean they could perform regulatory functions? Studies of human tissue immunology is very challenging, but the field is finding new ways to explore this exciting area and one can only be thrilled at the discoveries yet to come.

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